E. R. SQUIBB

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METABOLIC PATHWAYS IN MICROORGANISMS

V. H. CHELDELIN

Metabolic Pathways in Microorganisms

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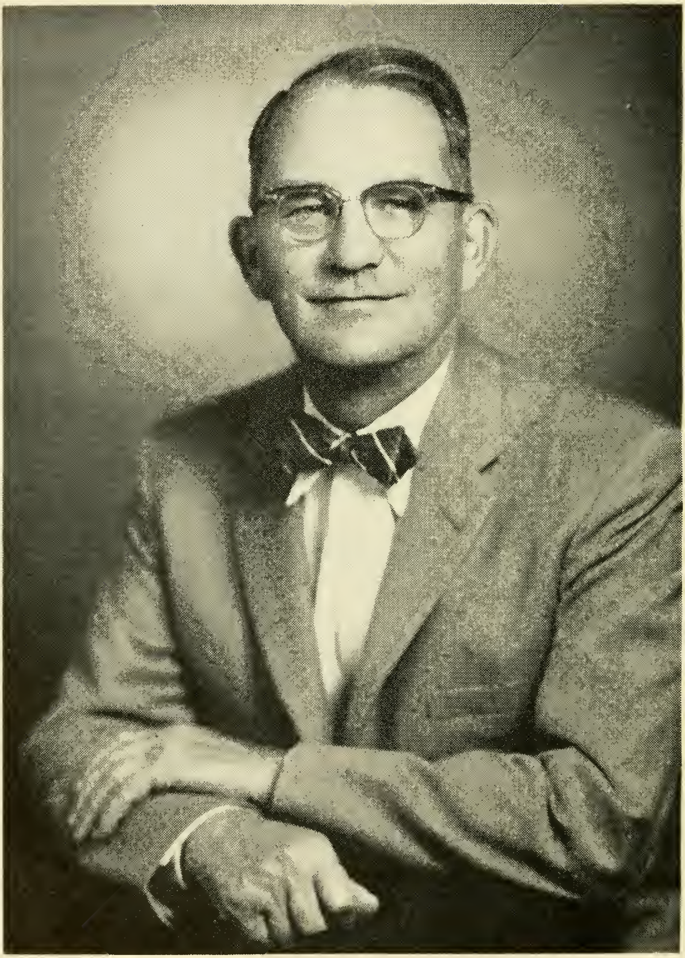

Roger J. Williams

CHEMISTRY OF MICROBIAL PRODUCTS

Metabolic Pathways in Microorganisms

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PREFACE

I am happy to have the opportunity to present this work on pathways of carbohydrate metabolism, which has been carried out, for the most part, in our laboratories (Science Research Institute, Oregon State University) during the last eight years. This work emphasizes heavily the peculiarities of metabolism that characterize the acetic acid bacteria, as well as emphasizing observations on microbial systems, which lend themselves especially to radiorespirometric studies. Finally, it presents a general discussion on various aspects of metabolism, which seems appropriate in this series of lectures. Because of the personal nature of the experiences described, this will not represent an attempt to review exhaustively the literature in the field, although it is hoped that this work may be of some aid in that direction.

Being selected as a lecturer to continue the series ably begun by the other speakers in the Squibb series produces in one mixed feelings—diffidence, yet at the same time the need for confidence to provide some useful thoughts about the subject chosen for discussion. The first reaction is spontaneous, and is so well recognized by everyone as to require no further elaboration. In marshaling my confidences to present this material, I am reinforced by some of the thoughts laid before me in my graduate study by Professor Roger J. Williams, to whom these lectures are dedicated, plus the sound, carefully fabricated experimental work by many of my colleagues over a period of fifteen years. Among these are Drs. Tsoo E. King, Chih H. Wang, and R. W. Newburgh, to mention only three, and also many former graduate students who have contributed to the over-all knowledge about the problem, especially Drs. Jens G. Hauge, Paul A. Kitos, and Joseph T. Cummins. To these persons, and others of our group, I am deeply grateful.

V. H. Cheldelin

August, 1961

CONTENTS

CHAPTER

1

THE ACETIC ACID BACTERIA

These bacteria, like several other species of microorganisms, have been utilized by man since antiquity; wild cultures invade cider, for example, to promote the acetic acid fermentation, and thus have been responsible for commercial vinegar production.

Acetobacter suboxydans, one of the better known members of this group, was discovered in 1924 by Kluyver and de Leeuw (1). Its discovery did much to broaden the industrial uses for the acetic acid bacteria, for in addition to the vinegar fermentation it carried out several others, mostly one- or two-step oxidations of polyols (2):

Mannitol → fructose (1)
Sorbitol → sorbose (2)
Glycerol → dihydroxyacetone (3)
Erytheritol → erythrulose (4)
Glucose → gluconic acid → ketogluconate (5)
2,3-Butanediol → acetoin (6)

as well as several others. The organism was at first regarded as a most versatile one, being adaptable to so many substrates; but, it may be more nearly correct to consider it as a metabolic "cripple," not particularly better able to attack substrates than many other organisms, and unable to oxidize these beyond the first one or two steps. Perhaps the chief reason for this is the fact, which will be discussed later, that the organism has no Krebs cycle, and thus lacks the "prairie fire" of terminal oxidation that most other organisms, whether men or mice, enjoy.

Our interest in this organism began, however, not with the oxidations that it carried out, but with finding a reason for its unusual pantothenic acid requirement. An earlier lecturer in this series, Dr. Frank M. Strong (3), has described much of the literature dealing with coenzyme A, so I will dwell on it only for a moment. Suffice it to say here that this organism is ten to twenty times as sensitive to bound forms of pantothenic acid [coenzyme A, pantetheine (*L. bulgaricus* factor, LBF), pantothenyl cysteine] as it is to the free vitamin (4). This fact was discovered in our laboratory, where it gave rise to the description of a pantothenic acid conjugate which we abbreviated PAC (5). This conjugate was not fully characterized, but it is now regarded as a fragment of the coenzyme A molecule. The enhanced activity of conjugates of pantothenic acid toward *A. suboxydans* has been of aid to various investigators in their studies of derivatives of pantoic acid that lead to coenzyme A (3, 6, 7).

The observed superiority of coenzyme A over the free vitamin as a growth promoting agent, may be rationalized by the fact that cells grown deficient in pantothenate (and hence coenzyme A) have a lower lipid content than normal cells. In view of the known function of this coenzyme in fat formation (8) this seems reasonable. Other activities, such as glucose oxidation, appear not to be influenced by coenzyme A deficiency, although the oxidation of glycerol is markedly reduced (see Fig. 1.1) (9) in a manner that is not yet understood.

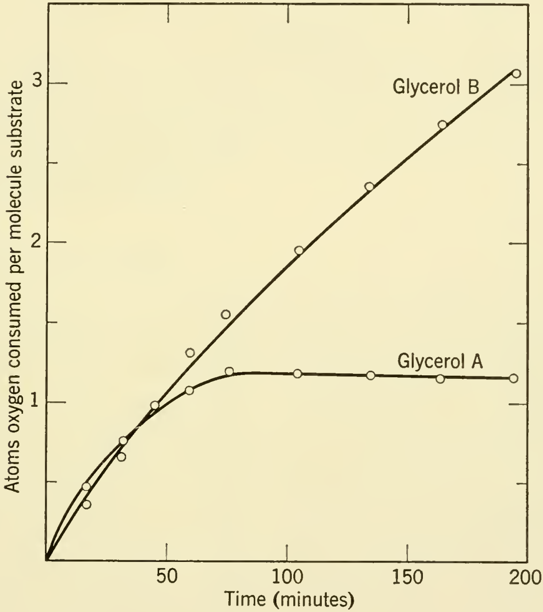

Fig. 1.1. Glycerol oxidation by pantothenate-deficient *A. suboxydans* cells. Deficient cells were grown in 0.0006 γ pantothenic acid equivalent per milliliter of medium. This concentration permitted growth of only 0.33 g. per liter, in contrast to sufficient cells which were grown in excess CoA and yielded a crop of over 1 g. per liter.

CARBOHYDRATE AND POLYOL OXIDATIONS

Testing of various carbon compounds soon revealed that several were inert, whereas others were susceptible to attack to only a limited degree. Table 1.1 indicates the extent of such oxidations. Where extensive oxidation oc-

TABLE 1.1

Oxidation of Various Substrates by *A. suboxydans*

	O_2 Consumption: μatoms/μmole	
	Dinitrophenol Addition	
Substrate	0	$1 \times 10^{-4}M$
Glycerol	3.7	1.0
Dihydroxyacetone	3.0	0.3
Ethanol	1.9	2.0
Acetaldehyde	1.0	
Sorbitol	4.0	1.2
Erythritol	1.0	
Pyruvate	0.9	
Lactate	2.0	
Acetate	0	
Ketoglutarate	0	
Malate	0	
Succinate	0	
Fumarate	0	
Citrate	0.4	

The systems contained $0.05M$ phosphate, $0.01M$ $MgCl_2$, $10^{-4}M$ DPN, and 10 mg. dry weight of washed cells. Volume = 2.8 ml., pH = 6.0, temperature = 29° C. The substrate was tipped into the main compartment containing dinitrophenol ($10^{-4}M$) after 5 minutes' preincubation. All values corrected for endogenous blanks, which were about 0.1 μatom oxygen/μmole substrate.

curred, as in glycerol or sorbitol, this could be reduced to one atom of oxygen per molecule of substrate by including dinitrophenol in the medium. The further oxidation of the one-step oxidation products (dihydroxyacetone or sorbose) was virtually completely repressed in dinitrophenol solutions, which presumably prevented coenzyme-linked phosphorylations.

The cells were therefore broken, and cell-free preparations were made. This was accomplished either by disintegrating the cells in a 10-kc. Raytheon sonic oscillator or by grinding with alumina. The broken cell suspension was mixed with phosphate buffer and centrifuged at 20,000*g* for an hour; then the residue was re-extracted and the combined extracts pooled.

Particulate Enzymes

These extracts revealed that the oxidizing enzymes of *A. suboxydans* varied considerably, more than had been suspected. Two, and sometimes three, systems existed side by side in the organism for the breakdown of individual polyhydroxy compounds. There were, for example, a number of particle-bound, phosphate-independent dehydrogenases (10) which oxidized mannitol, sorbitol, erythritol, glycerol, and glucose to the extent of one atom of oxygen per molecule of substrate. Two atoms of oxygen were used per molecule of ethanol or propanol. The dehydrogenases appeared different from each other, since purification of the particulate suspensions effected a ten-fold increase in the concentration of glucose dehydrogenase (oxidase), four-fold for erythritol, three-fold for glycerol, but did not affect the concentration of ethanol dehydrogenase.

δ-D-Gluconolactone has been indicated as the product of

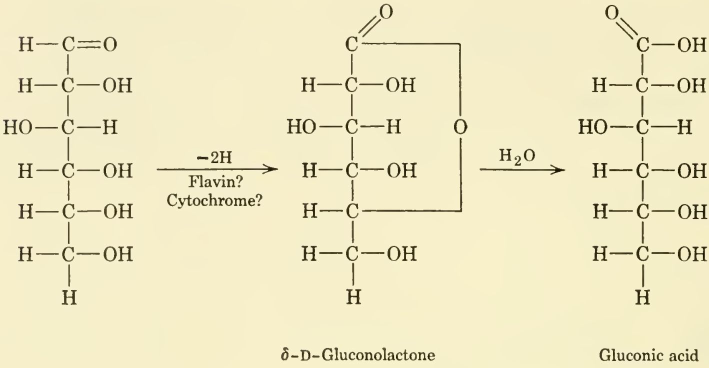

Fig. 1.2. Oxidation of glucose by particulate glucose oxidase in *A. suboxydans.*

D-glucose oxidation by a particulate enzyme (see Fig. 1.2). The optimum pH was 5.5 (11). A separate enzyme also exists in this fraction, which hydrolyzes the lactone to D-gluconic acid. Deoxycholate extracts of the particulate fraction retained activity for oxidation of glucose, but they no longer contained the hydrolyzing enzyme.

"Soluble" Enzymes

In addition, the cell-free extracts contained several *soluble* enzymes that cooperate to effect the *terminal* oxidation of glucose, other carbohydrates, and polyols. These were, for the most part, phosphate-dependent and DPN- or TPN-linked. Upon fractionation, the extracts were found to contain the entire pentose cycle complex of enzymes (Fig. 1.3).

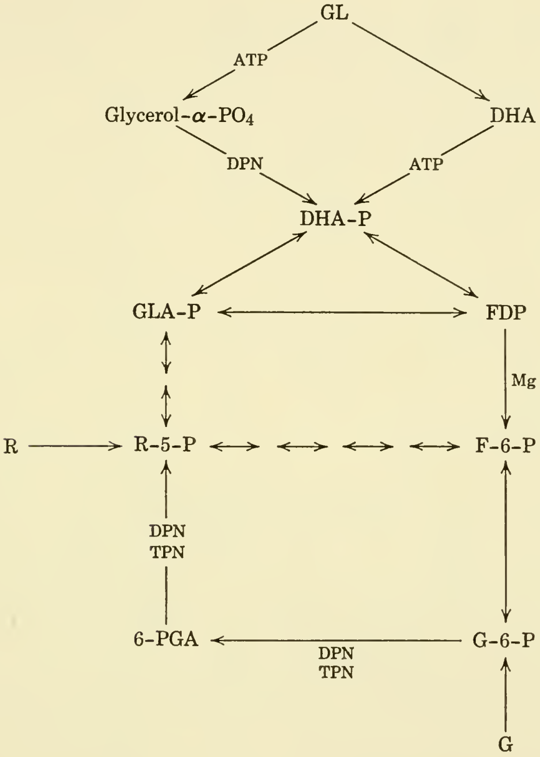

Fig. 1.3. The pentose cycle in *A. suboxydans.*

Glucose. The first example of a soluble dehydrogenase is an exception to the generalization just stated: phosphate does not participate in glucose oxidation to gluconate, though pyridine nucleotide (TPN) does. The soluble dehydrogenase catalyzing this oxidation is a separate enzyme from the particulate enzyme already discussed, since it has its pH optimum at 8.6. We have purified this enzyme about 100-fold and have found it to be strictly TPN-specific. The reaction is reversible, as tested with δ-D-gluconolactone and TPNH, but the γ-lactone is less active. Among the sugars and their phosphates tested, only D-glucose and 2-deoxy-D-glucose were oxidized, whereas glucose-6-phosphate, glucose-1-phosphate, gluconolactone, and gluconic acid were not attacked. This enzyme therefore appears different from those described by de Ley and Stouthamer (12) for this organism in the oxidation of gluconate.

Glucose-6-Phosphate and 6-Phosphogluconate. These two soluble enzymes usually are closely associated in *A. suboxydans,* as they are in most organisms. They have been highly purified, to the point that they catalyze the disappearance of about 100 and 50 μmoles of substrate per minute, respectively, per milligram of enzyme. With a pH optimum at 8.0, the turnover numbers for G-6-P dehydrogenase are about equal with DPN and TPN, whereas DPN is much superior with 6-phosphogluconic dehydrogenase. The purifications are effected by combinations of protamine precipitation, ammonium sulfate fractionation, calcium phosphate gel adsorption, and column electrophoresis (13). A comparison of properties of G-6-P dehydrogenase from various sources is given in Table 1.2, whereas Table 1.3 contains a similar listing of properties of 6-PGA dehydrogenases.

For clarification, the enzymes that oxidize glucose or glucose-6-phosphate, as reported from different laboratories, are listed in Table 1.4 along with some of their distinguishing properties. It is evident from Table 1.4 that *A. suboxydans* is well endowed with separate enzymes all of which attack these substrates. The reasons for this diversity are not yet clear. It is possible that there is little or no

TABLE 1.2

Comparison of Characteristics of Glucose-6-Phosphate Dehydrogenase from Different Sources

Source	Coenzyme Specificity	Fold Purification	Highest Specific Activity *	Optimum pH	Substrate K_m	Reference
Yeast	TPN, Mg, or Mn	600–900	86	8.5	$2.0\text{–}6.9 \times 10^{-5}$	14
E. coli	TPN, Mg	11	7	7.7–8.6	3×10^{-4}	15
A. suboxydans	TPN or DPN	100	100	8.0	3.9×10^{-4} (DPN)	
Mammary gland	TPN	10,000 (crystalline)	420			16

* 1 unit = amount causing 1.0 optical density change at 340 mμ. Specific activity = units/minute/milligram protein.

TABLE 1.3

Comparison of Characteristics of 6-Phosphogluconate Dehydrogenase from Different Sources

Source	Coenzyme Specificity	Separation of 6-PGA and G-6-P Dehydrogenases	Highest Specific Activity *	Optimum pH	Substrate K_m	Reference
Yeast	TPN	30% G-6-P dehydrogenase	15.6	7.4	5×10^{-5}	17
Leuconostoc	DPN		2.0	7.8	7.8×10^{-5}	18
E. coli	TPN	4% G-6-P dehydrogenase	2.7	6.6–7.7	3×10^{-5}	15
A. suboxydans	TPN or DPN	Complete	50	8.0	2.3×10^{-4} (DPN) 1.3×10^{-3} (TPN)	

* 1 unit = amount causing 1.0 optical density change at 340 mμ. Specific activity = units/minute/milligram protein.

TABLE 1.4

Distinguishing Features of *A. suboxydans* Enzymes Oxidizing Glucose or Glucose-6-Phosphate, Gluconate or 6-Phosphogluconate

Substrate	Source	Coenzyme Specificity	Product	Optimum pH	Reference
Glucose	Particulate fraction	None reported	δ-Gluconolactone	5.5	11
Glucose	Soluble fraction	TPN	δ-Gluconolactone	8.6	11
Glucose-6-PO_4	Soluble fraction	TPN, DPN	6-PO_4 gluconate *	8.0	13
Gluconate	Particulate fraction	None reported	2-Ketogluconate	—	12
Gluconate	Soluble fraction	TPN	2-Ketogluconate	—	12
Gluconate	Soluble fraction	TPN	5-Ketogluconate	—	12
6-PO_4 gluconate	Soluble fraction	DPN, TPN	Pentose phosphate	8.0	11, 13

* Presumably the lactone is the preliminary product.

free passage of glucose or its phosphate from one oxidizing system to another, yet this implies that no "pool" of glucose derivatives exists. While this has neither been proved nor disproved, it seems not to be a tempting conclusion. In the organism *B. subtilis,* kinetic experiments with isotopically labeled gluconate suggest strongly that a "pool" of glucose exists, and that gluconate when reconverted to hexose via the pentose cycle is labeled in accordance with expected patterns, as will be seen in the next chapter. Spatial separation might seem to provide a plausible explanation for the plethora of separate enzymes that exists for oxidation of individual, or closely related, substrates.

Dihydroxyacetone Phosphate. Dihydroxyacetone phosphate was converted, after its formation from glycerol, to fructose 1, 6-diphosphate through the action of the isomerase-aldolase system, which was found to be very active in this organism (19). With dihydroxyacetone as the starting material, it was possible, in the presence of ATP and Mg^{2+}, to show the formation of fructose in amounts (as measured by the resorcinol test) approaching theory based on the ATP present, assuming activity of ADP. The reactions could also be followed through measurement of the inorganic phosphate released. Under oxidative conditions (DPN and triphenyltetrazolium) a further, slower release of P_i was observed, as would be expected if the pentose cycle were operating, and hexose accumulation dropped to about one-tenth of the amount formed non-oxidatively. In the presence of Mg^{2+}, a material was formed that produced two paper chromatogram spots characteristic of an authentic mixture of glucose-6-P and fructose-6-P. The scheme is shown in Fig. 1.3, together with subsequent reactions of the pentose cycle.

When the oxidation of glucose-6-phosphate was followed manometrically (by the cell-free extract), the CO_2 production lagged initially, compared to the O_2 consumption. Chromatography of the oxidation products revealed zones corresponding to 6-phosphogluconate and ribose-5-phosphate. Both DPN and TPN appeared active in the dehydrogenases for glucose-6-phosphate and 6-phosphogluconate (see Table 1.4).

The transketolase-transaldolase reactions that characterize the pentose cycle were demonstrated by chromatography and the appropriate color reactions. Sedoheptulose was determined by the cystine–sulfuric acid reaction, at 415 mμ and 505 mμ, and ribose was measured with orcinol. Sedoheptulose and the phosphates of fructose, glucose, and dihydroxyacetone were also measured chromatographically. The recovery of total sugar and the measurement of each sugar derivative with time is shown in Table 1.5, where ribose-5-phosphate is added non-oxidatively to the cell-free extract and its rate of disappearance is followed together with appearance of other sugars.

Other ancillary reactions leading into the pentose cycle, such as kinases for ribose, erythritol, and glucose, have been identified. The glucokinase has been partly purified (19).

Each reaction of the pentose cycle, plus related ones listed in Fig. 1.4, has thus been documented in *soluble* extracts of the organism. The quantitative importance of the pentose cycle as a terminal respiratory mechanism in *A. suboxydans* has been demonstrated (23) through the use of specifically C^{14}-labeled glucose and gluconate as substrates for aerated resting cells: for every 100 molecules administered 28 were oxidized to 2-ketogluconate, presumably by the particulate dehydrogenases. Of the remaining 72, 63 (equals 88%) entered the pentose cycle. As a later chapter will reveal, we have calculated that essentially all

TABLE 1.5

Non-oxidative Breakdown of Ribose-5-Phosphate by *A. suboxydans* Extract

Time (Minutes)	0	2	5	20	80
Sugar	μMoles Sugar				
Pentose	10.0	8.9	7.3	4.4	0.8
Hexose	0.0	0.0	1.1	2.6	4.7
Sedoheptulose	0.0	0.4	0.7	0.9	0.6
Triose-phosphate	0.0	0.5	0.6	0.6	0.8
Total *	10.0	9.8	10.0	9.1	7.8

* Values for total sugars are given as pentose equivalents (μmoles carbon/5).

The tubes were incubated *in vacuo* with 10 μmoles ribose-5-phosphate, 100 μmoles tris buffer at pH 8.0, 20 μmoles sodium fluoride, 0.15 ml. cell-free extract, and water to 2 ml. The reaction was stopped by adding 1.6 ml. of 10% trichloroacetic acid. Pentose was assayed in the reaction mixtures according to Mejbaum (20), hexose and sedoheptulose with the sulfuric acid–cysteine reaction (21), and triose-phosphate as alkali-hydrolyzable phosphate. Magnesium was omitted. [After Hauge et al. (22).]

of the CO_2 produced from glucose arises via the pentose cycle; in fact, *A. suboxydans* is unique in this respect among organisms studied to date. The active existence of the pentose cycle in this organism makes more reasonable the finding that the Krebs cycle appears absent. This latter difference is the first clear one between *A. suboxydans* and related *Pseudomonas* species, several of which rely heavily on the Entner-Doudoroff pathway for glucose breakdown (24).

Other results, summarized in Fig. 1.4, which deserve special mention are the following.

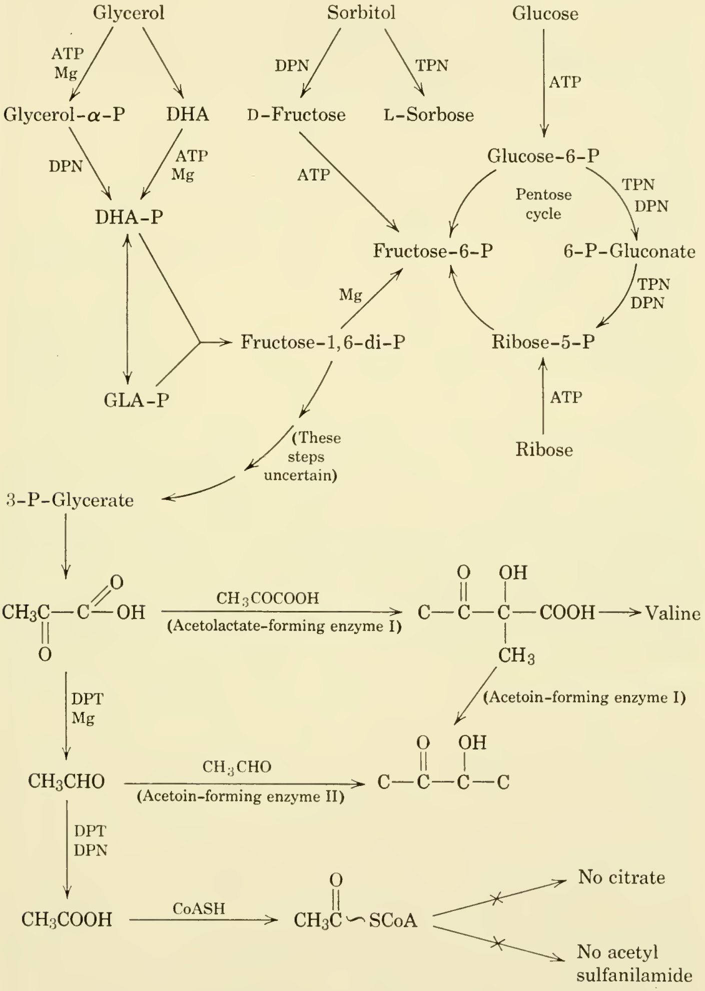

Fig. 1.4. Carbohydrate dissimilation in soluble extracts of *A. suboxydans.*

Glycerol. Glycerol enters the pentose cycle via dihydroxyacetone (Figs. 1.3 and 1.4). This conversion is reached by two alternate pathways (25), both of which are in the soluble fraction (soluble at 30,000*g*). One, active at pH 6.0, is independent of ATP and DPN, and yields dihydroxyacetone directly. The other, with a pH optimum around 8.5, requires the participation of ATP and Mg^{2+}, in a kinase reaction, to yield glycerol-α-phosphate. The latter is then oxidized by a DPN-dependent dehydrogenase to form $DHA\text{-}PO_4$.

Sorbitol. Sorbitol (Fig. 1.4) is oxidized on either end of the molecule by soluble extracts of *A. suboxydans,* depending upon which pyridine nucleotide is present (26, 27). In the presence of TPN, L-sorbose is formed; with DPN, D-fructose is produced (see Table 1.6). The fructose can then be phosphorylated or further oxidized via the pentose cycle. These two pathways occur *in addition* to the previously demonstrated sorbitol dehydrogenase in the particulate fraction of the cells, which form L-sorbose.

The DPN-linked enzyme has been purified about sixteenfold so that it is free from the TPN (sorbose-producing) enzyme, and is also free of mannitol dehydrogenase activity (27). An effective purification step consists of heating the enzyme in the presence of a polyhydroxy compound and pyridine nucleotide; the sorbitol dehydrogenase is protected by these compounds, while many other proteins are denatured and may be removed by centrifuging.

Although earlier work (30, 31) has suggested the presence of some relatively non-specific polyol dehydrogenases in mammalian tissues, the present experience with *A. suboxydans* indicates a higher degree of specificity at least as far as sorbitol is concerned. The purified DPN enzyme, active for sorbitol, was completely inactive toward mannitol,

TABLE 1.6

Identification of Products of Sorbitol Oxidation by *A. suboxydans*

	Pyridine Nucleotide Added		D-Fructose Standard	L-Sorbose Standard
	DPN	TPN		
Position constant in phenol-H_2O (4:1) *	0.93	0.69	1.00	0.73
$[\alpha]_D^{20}$ (28), degrees	−102	−48	−92	−42
Crystal form of osazone (29)	Rosettes	Amorphous	Rosettes	Amorphous
Melting point of osazone (28), °C.	206	161.5–163	206	163

* The position constant is the distance traveled by the compound divided by the distance traveled by fructose.

The reaction mixtures contained the following: 180 μmoles of sorbitol, 10 μmoles of TPN or DPN, 3 ml. of CFE, 100 μmoles of TTZ, 500 μmoles of $MgCl_2$, 1 mmole of tris buffer, pH 8.5. The total volume was 10 ml.; temperature, 30°; time, 4 hours. 1 ml. of 50% TCA was added, and the mixture was centrifuged and extracted with ether. The $[\alpha]_D^{20}$ was calculated on the basis of sorbose and fructose determined both by the cysteine-H_2SO_4 and resorcinol methods. [After Cummins et al. (26).]

ribitol, dulcitol, perseitol, glycerol, ethanol, acetaldehyde, or 2-butene-1, 4-diol. Mannitol was oxidized as extensively as fructose by whole cells (8.7 atoms of oxygen per molecule substrate) but this may reflect only the easy conversion of mannitol to fructose by this organism. Mannitol oxidation has been found in our laboratory to be completely TPN-specific and completely separable from the TPN-sorbitol enzyme.

The influence of pyridine nucleotide in guiding the oxidation toward one end or the other of the sorbitol molecule is unusual, although a related directing of metabolism toward glycolysis (DPN) or the pentose cycle (TPN) has been noted in animal systems in our laboratory (32), as well as by Wenner and Weinhouse (33).

Pyruvate. Pyruvate conversion to acetate in *A. suboxydans* follows an interesting course (34, 35). Whereas in animals and most other aerobic organisms, the highlight of pyruvate oxidation is terminal consumption through the Krebs cycle, in this organism there is ample opportunity to recognize breakdown products from pyruvate that have more than a transient existence. The details of formation of some of the minor products also differ. Although in animals and most bacteria, pyruvate is converted in small

TABLE 1.7

Comparison of Substrate Specificity of *A. suboxydans* and Yeast Pyruvic Carboxylase

		Yeast *	
Acid Added	*A. suboxydans*	Green et al. (36)	Kobayasi (37)
Pyruvic	1.00	1.00	1.00
α-Ketobutyric	0.75		0.80
α-Ketoglutaric	0.00	0.01	0.04
α-Ketoisovaleric	0.00	0.88	0.26
α-Ketoisocaproic	0.00	0.05	
Oxalacetic	0.7	0.32	0.54
Phenylpyruvic	0.00		0.00

* Recalculated from the data of Green et al. (36) and Kobayasi (37).

A direct quantitative comparison is not possible because of differences in test conditions. The numbers represent relative rates of decarboxylation compared to the rate with pyruvic acid. [After King and Cheldelin (34).]

measure to acetoin, in *A. suboxydans* pyruvate is first decarboxylated to acetaldehyde and the latter compound is then oxidized to acetate. *A. suboxydans* is thus one of a very few with a yeast-type decarboxylation (also shared by higher plants); it is probably associated with the failure of the organism to form acetyl phosphate or acetyl CoA in this organism (see below).

Pyruvic carboxylase has been prepared from this organism, in a high state of purity (turnover = 300 moles CO_2 produced/minute/100,000 g. of enzyme). Thiamin pyrophosphate and a divalent ion serve as cofactors. Such preparations show a higher degree of specificity than does yeast carboxylase, since the bacterial enzyme responds only to α-ketobutyrate, oxalacetate, and pyruvate, as shown in Table 1.7.

Acetaldehyde. The oxidation of acetaldehyde may proceed, as do several other oxidations in *A. suboxydans,* by two routes: one with TPN as coenzyme (more active), the other with DPN, although it has not been possible to determine with certainty whether two apoenzymes are present (35). The specific activity of the purest preparations [about 140 spectrophotometric "units" (Table 1.2)] is considerably higher than any reported for yeast or liver.

Acetoin. Acetoin formation (38), as depicted in Fig. 1.4, has revealed that this metabolite appears to arise differently from pyruvate, acetolactate, or from acetaldehyde. We believe that two acetoin-forming enzymes may be present: one that employs acetolactate as the preferred substrate, and the other which employs acetaldehyde. A final decision on this point will probably have to await further fractionation of the partially purified enzymes.

Acetate. The utilization, or rather non-utilization of acetate by *A. suboxydans* has received much study. In addition to the stoichiometry already quoted (23) which demonstrates the preponderant use of the pentose cycle for terminal oxidation of carbohydrate, there are several other lines of evidence which indicate that acetate cannot be oxidized by this organism. These are:

1. Added acetate is not oxidized in simple manometric experiments by intact cells or by cell-free extracts, either alone or in the presence of glucose or glycerol as a potential "sparker."

2. When $CH_3C^{14}OOH$ is added to respiring cells, only 0.013% of the added C^{14} appears in the respiratory CO_2, even under conditions where 25% of the added C^{14} is incorporated into the lipid fraction of the cells (see Table 1.8) (39).

3. *A. suboxydans* extracts can form acetyl CoA in good yield by the ATP-acetate-CoA reaction, yet the acetyl CoA formed cannot be converted to citrate or acetyl sulfanilamide. The organism evidently lacks a suitable acceptor system; only when the acceptor fraction from pigeon liver [Chou and Lipmann (40)] is added can acetyl sulfanilamide be produced, and only when pigeon liver condensing enzyme is added can citrate be formed.

4. Pyruvate does not form acetyl CoA during oxidation unless ATP is added (39). This suggests that free acetate is produced before acetyl CoA is formed.

The Citric Acid Cycle. The statements in the four preceding paragraphs imply that the Krebs citric acid cycle may not function in *A. suboxydans.* This is of course surprising, for other species of *Acetobacter* such as *A. pasteurianum* (41) and *A. aceti* (42) have been shown to possess a full complement of Krebs cycle enzymes, as indeed virtually all

TABLE 1.8

Fate of C^{14} from $CH_3C^{14}OOH$ Administered to Resting Cells of *Acetobacter suboxydans*

			Radioactivity Distribution					
	$CH_3C^{14}OOH$ Added		Respiratory CO_2		Medium		Cells	
Experiment Number	Moles	Total CPM	CPM	% of Total	CPM	% of Total	CPM	% of Total
1	6	3×10^6	401	0.013	1.67×10^6	56	7.62×10^5	25.4
2	200	5×10^5	10	0.002	3.16×10^5	63	10	0.002

Each vessel contained 250 mg. dry weight of fresh *A. suboxydans* cells in a 500-ml. three-necked standard taper flask. Composition of medium, 0.4% monopotassium phosphate, pH 6.0. Volume = 100 ml. CO_2-free air was bubbled through the medium for 60 minutes before $CH_3C^{14}OONa$ was added to the vessels. Experiment 1: 6 μmoles $CH_3C^{14}OONa$, specific activity 5 μc./μmole. Experiment 2: 200 moles $CH_3C^{14}OONa$, specific activity 0.025 μc./mole. In both experiments 250 μmoles glucose were added after 2 hours. The metabolic CO_2 was trapped by sparging through 0.5*N* NaOH. The NaOH traps were replaced periodically and the CO_2 was plated as $BaCO_3$, weighed, and counted. [After Kitos et al. (39).]

organisms do. Moreover, the idea that such a universally important complex of metabolic machinery should be completely absent from any aerobic organism that can oxidize glucose to CO_2 and H_2O, is not likely to gain full acceptance—even by those who work with the organism—without careful checking. We have scrutinized this problem carefully, but all data point to the absence of a traditional citric acid cycle in *A. suboxydans* [to the extent that these reactions have been studied, they have in general been verified by Rao (42)]. The pertinent data are:

1. Failure to form citrate from acetyl CoA, as outlined in part 4 of the previous section.

2. Failure of either whole cells or cell-free extracts of *A. suboxydans* to oxidize, succinate, fumarate, malate, or α-ketoglutarate. Oxalacetate is oxidized to pyruvate and acetate. The only other citric acid cycle member to be oxidized at all is citrate, and this only slightly. The possible significance of this oxidation is being examined further.

3. The quantitative data on $C^{14}O_2$ arising from cells oxidizing glucose- or gluconate-1-, 2-, 3,4-, 6-, or U-C^{14} indicate that the pentose cycle is the terminal oxidation route in this organism. There is no indication that glycolysis or the Krebs cycle are operative (23).

Glycolysis. *A. suboxydans* displays high aldolase and triose phosphate isomerase activity, and might be supposed to carry out glycolysis in the usual manner. However, the failure to produce significant amounts of acetate from glucose (lactate and pyruvate are quantitatively converted to acetate) has cast doubt upon the presence of a typical Embden-Meyerhof dissimilation scheme. The presence of individual enzymes characteristic of glycolysis does not of itself assure glycolytic action, since *every* enzyme in the scheme except phosphofructokinase may also be used by either the pentose cycle or the Entner-Doudoroff pathway.

The isotope data of Kitos et al. (23) argue strongly against the use of glycolysis in whole *A. suboxydans* cells. Likewise, when extracts are employed to oxidize glucose or glycerol, the pentose cycle prevails (no acetate is formed unless lactate or pyruvate is added). However, when the cell-free extract is treated with Dowex-50 to remove Mg, DPT, and pyridine nucleotides, it becomes possible to show fructose diphosphate disappearance in a DPN-dependent reaction, and the accumulation of 3-hour stable phosphate, suggestive of 3-PGA formation. Both aldolase and triose phosphate dehydrogenase are active under these conditions (43). It thus appears that at least some of the reactions of glycolysis can function in the traditional manner when external conditions permit (or force?) their operation. The importance of Mg in this test is clearly evident; in its presence the pentose cycle is favored to the virtual exclusion of glycolysis.

THE BIOSYNTHESIS OF AMINO ACIDS

The foregoing sections on acetate non-utilization and lack of a citric acid cycle logically raise the question of the origin of amino acids that normally arise from the cycle, especially glutamate and aspartate. These reactions are under study, although at present the crucial experiments remain to be carried out. Several bits of information are known —for example, that this organism has great synthetic powers, requiring [in contrast to the findings from an earlier study (44)] only serine plus either glutamate, histidine, or proline for healthy growth, with glycerol as substrate. [When glucose is the carbon source, serine may be omitted (45).] The earlier report (44) listed valine as an essential growth substance, but we have found it to be formed from acetolactate, probably similarly to the formation of this amino acid in yeast (46). When isotopic glucose is administered, the label appears first (among amino acids) in aspartate and glutamate; the same is true of administered $C^{14}O_2$. Also (although this may not be related) despite the fact that acetate is not oxidized by *A. suboxydans,* $CH_3C^{14}OOH$ can nonetheless give rise to labeled succinate.

At one point we had surmised that the isocitrase reaction (Fig. 1.5) (47) might be operative in *A. suboxydans,* and that it might aid in isocitrate formation by acting in reverse to form isocitrate from glyoxylate + succinate, or even to form oxalsuccinate from glyoxylate + malate. However, although one successful experiment (48) was performed that tended to support this idea, subsequent efforts have failed. What has been found instead is that glyoxylate has only

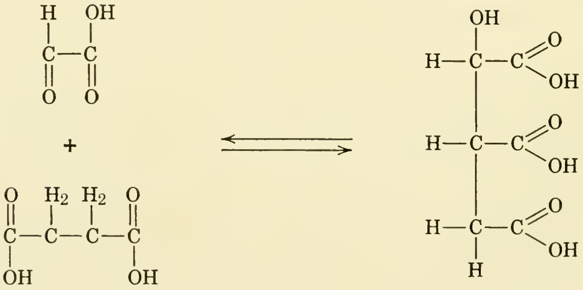

Fig. 1.5. The isocitrase reaction.

a transient existence, due to the presence of a very active glyoxylate *reductase* (glycolic dehydrogenase) in the organism (49). This enzyme, studied in detail in plants by Zelitch (50), has an equilibrium constant of 1×10^{-13} in the direction glycolate → glyoxylate; hence only traces of glyoxylate could be present at any moment.

PHOSPHORYLATION

The non-phosphorylative oxidations of this organism presumably proceed without capture of the released energy, at least as nucleotide polyphosphates. These oxidations are not inhibited by dinitrophenol (51). In contrast, the oxidations beyond the first step, i.e., those in the pentose cycle, are phosphate dependent and, as mentioned earlier, are dinitrophenol sensitive.

This observation prompted a more complete investigation of oxidative phosphorylation in *A. suboxydans.* Using glucose, glycerol, and fructose as substrates and $P^{32}O_4^{3-}$ as a tracer, it was found (52) that oxidative phosphorylation in this organism differs in certain important respects from the corresponding process in animal tissues. Thus,

1. Unlike animal systems, but like many other microbial systems, the P/O ratios are low, averaging about 0.5.
2. The nucleotides that are active in phosphorylation are present in low concentrations (about 1.5 μmoles ATP per gram of respiring cells). It has not been possible to increase these values through the addition of acceptors.
3. Inorganic pyrophosphate is present in relatively large amounts (about one-eighth that of ATP) and turns over

rapidly. However, its specific activity does not reach that of orthophosphate, but levels off at about half that value.

4. After prolonged incubation, the specific activities of the labile groups in ATP and ADP approach that of inorganic *pyro*phosphate, not orthophosphate.

5. About five to ten times as much high polymer metaphosphate is found as is ATP. This material is slowly labeled by added $P^{32}O_4{}^{3-}$, although there is some evidence that metaphosphate may be formed in resting cells oxidizing fructose and glycerol.

These data point strongly to inorganic pyrophosphate as an intermediate in oxidative phosphorylation, in *A. suboxydans* as well as in yeast. The *origin* of pyrophosphate remains to be studied; it may arise through interaction of polymetaphosphate with $P^{32}O_4{}^{3-}$.

SUMMARY

The organism *A. suboxydans* thus presents a variety of behaviors that makes it seem curious indeed. Its special behavior toward glucose has made it possible to recognize a number of reactions, which, although present in other organisms as well, are easier to view here because of the absence of a functioning Krebs cycle. The Krebs cycle, if present, might serve to remove many of the metabolites that are now recognizable. The documentation of the pentose cycle, both with respect to its extent and its abundance, will be treated in the next chapter, where attention is paid to the complex problem of evaluating pathway participation in whole cells through the use of isotopes.

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CHAPTER

2

EVALUATION OF METABOLIC PATHWAYS

Our attention shifts in this chapter to a subject arising out of the previous discussion, namely, an answer to the question how to tell how much each given avenue of metabolism is employed in the normal cell. In other words, have traffic counting techniques been established, are they reliable, and what do the counts show? The answers to these questions are varied and almost too numerous; this tends to reflect incomplete satisfaction with many of the methods so far developed.

Metabolic inhibitors, or "traffic blocks," have been used from time to time to study metabolic pathways. They cannot be used satisfactorily to study the pentose cycle because suitable specific inhibitors of this cycle have not been developed. Beyond this, it is questionable whether their use would be warranted even if appropriate inhibitors were developed, since it cannot be readily determined whether the unphysiological conditions that would result from the accumulation of metabolites might introduce spurious readings of the "traffic count."

SPECIFIC ACTIVITY OF $C^{14}O_2$

In principle, a distinction among the pathways should be easy with isotopic tracers. As shown in Fig. 2.1, if a molecule of glucose tagged in carbon 1 undergoes glycolysis, the carbon chain is split in half; carbons 3 and 4 of glucose become the carboxyl group of pyruvate, carbons 2 and 5 become the carbonyl group, and carbons 1 and 6 the methyl

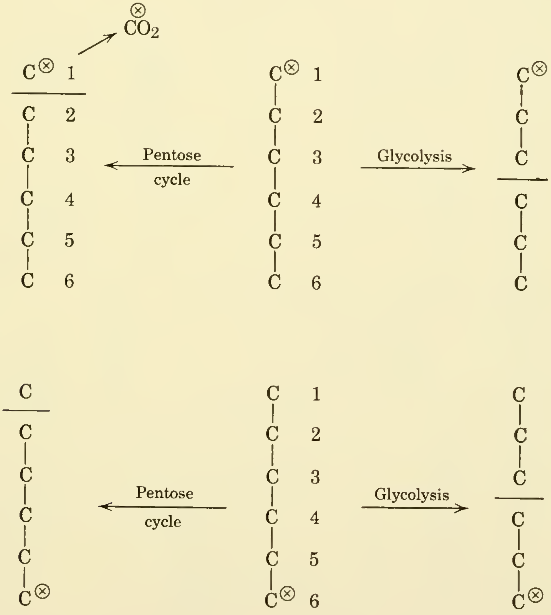


Fig. 2.1. Sketch of the routes of breakdown of glucose by glycolysis and by phosphogluconate cleavage.

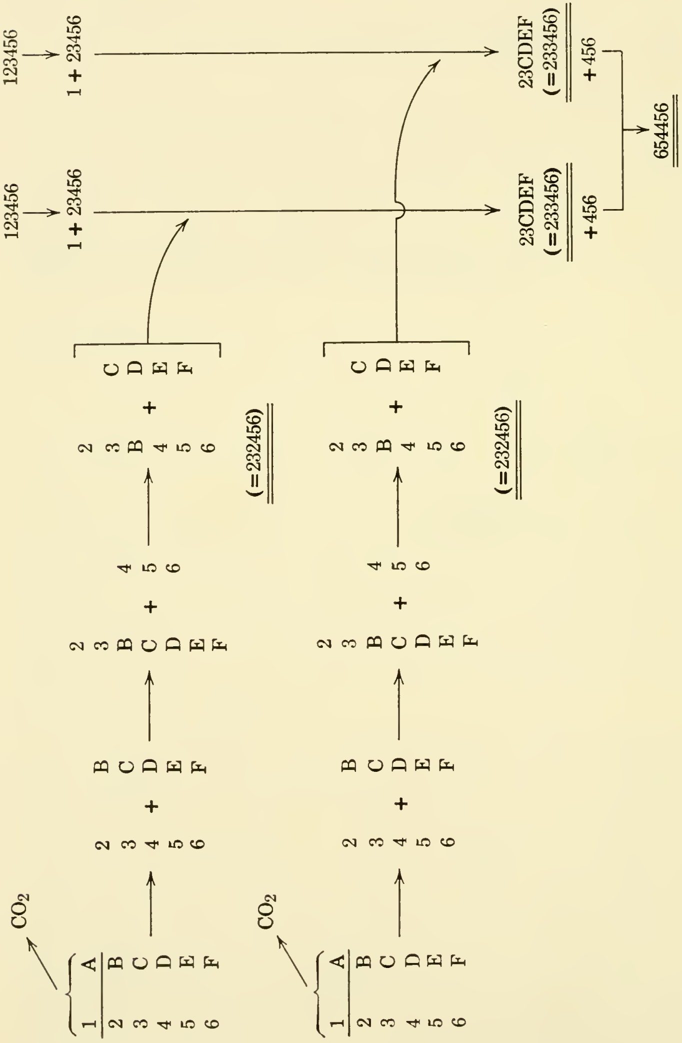


Fig. 2.2. The pentose cycle: fate of individual carbon atoms, according to Beevers (1).

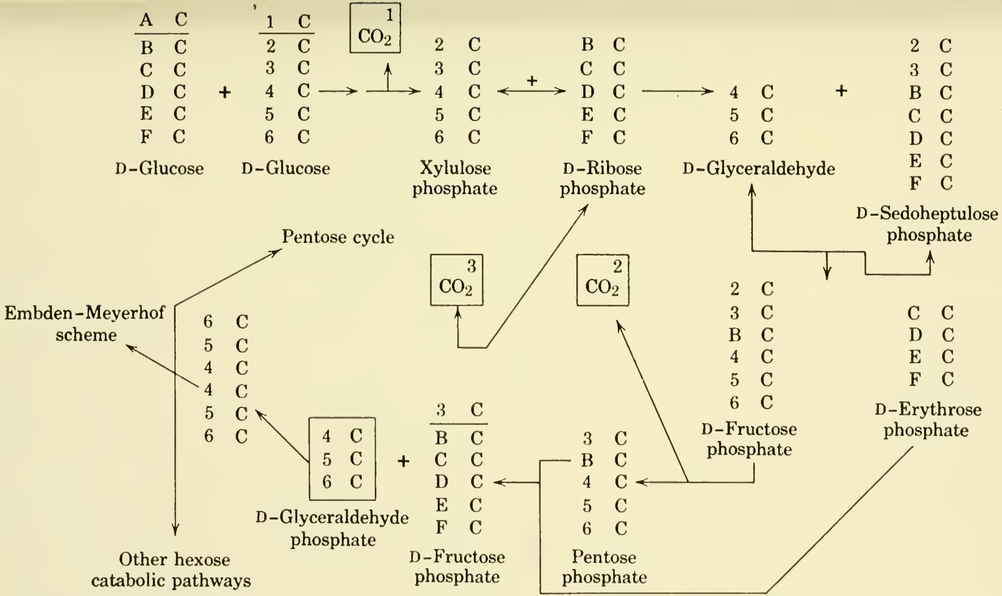


Fig. 2.3. The pentose cycle: fate of individual carbon atoms, according to Kitos et al. (4). This scheme varies from that of Beevers (Fig. 2.2) in that the molecule of tetrose, labeled CDEF, is here shown reacting with regenerated pentose from the transaldolase reaction; in Beevers' scheme the same tetrose CDEF reacts with "fresh" pentose from the cleavage of 6-phosphogluconate. The scheme of Beevers, first studied in plants (1) and later in *B. subtilis* by Wang et al. (11) appears to fit better the observed intramolecular isotopic distributions in regenerated hexose.

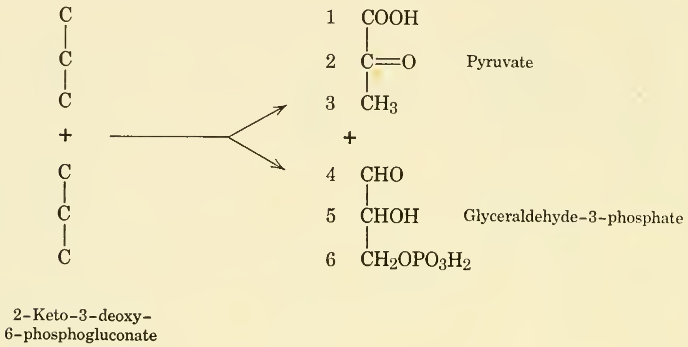


Fig. 2.4. The fate of the carbon skeleton of glucose in the Entner-Doudoroff pathway.

group. The specific activity of the respiratory $C^{14}O_2$ should be identical whether glucose-1-C^{14} or glucose-6-C^{14} is administered to the test system.

If, on the other hand, phosphogluconate decarboxylation prevails, carbon 1 of glucose will be quickly converted to respiratory CO_2. If this carbon is labeled, the CO_2 will be substantially enriched with C^{14}, whereas if carbon 6 is labeled instead, the CO_2 will have little or no activity at first, and will become more active only as the pentose cycle operates to relocate active carbon atoms into the oxidizable position of the glucose molecule. Figure 2.2 indicates the fate of individual carbon atoms as they traverse the cycle; this scheme has been reported by Beevers (1) for plant systems, and work in Dr. Wang's laboratory with *B. subtilis* oxidations indicates that the scheme outlined may operate in this fashion in this organism as well.[1]

[1] Beevers' scheme is slightly different from the one presented by Kitos et al. (4); see Fig. 2.3. The latter, which showed one molecule of hexose

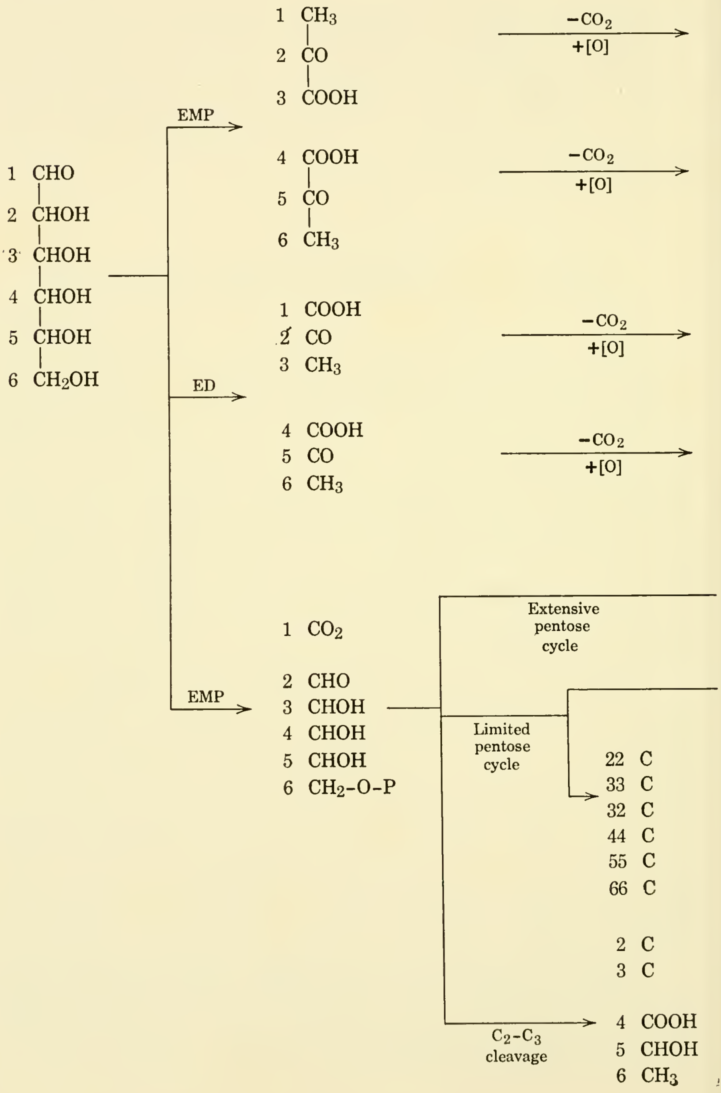
A third possibility involves the operation of the Entner-Doudoroff pathway (2) (Fig. 2.4). Here the yield of CO_2 will be identical from carbons 1 and 4 of glucose, from carbons 2 and 5, and from 3 and 6. Administration of glucose labeled specifically in these positions should reveal the presence of this route.

LABELING OF FERMENTATION PRODUCTS

Finally, use may be made of labeling of products isolated from fermentation or other degradation of glucose. Thus, Blumenthal, Lewis, and Weinhouse (3) have used the intramolecular distribution of isotope in acetate derived from labeled substrate as the criterion for identifying the metabolic routes that are being followed. Carbon 1 of glucose, for example, would be converted to carbon 2 of acetate by glycolysis, whereas it would not get into acetate at all via the pentose cycle; and so on. A summary of locations of specific glucose carbon atoms in various metabolic end products by different pathways is given in Fig. 2.5.

Actually, the evaluation of experimental findings is not quite so simple as might be expected from a cursory inspection of Fig. 2.1. Early workers, in comparing the specific activities of $C^{14}O_2$ arising from C-1 and C-6 of glucose, often found the value to be several-fold higher from C-1. The conclusion was reached that the major portion of glucose metabolism was via phosphogluconate decarboxylation. This, as is now recognized, was in error,

catalyzing the decomposition of all the others, seems attractive for this reason; however, the data of Beevers (1) as well as those to be presented below indicate that his suggestion (Fig. 2.2) should probably rate as the preferred mechanism.


1 CHO
2 CHOH
3 CHOH
4 CHOH
5 CHOH
6 CH2OH
EMP
1 CH3
2 CO
3 COOH
4 COOH
5 CO
6 CH3
ED
1 COOH
2 CO
3 CH3
4 COOH
5 CO
6 CH3
−CO2
+[O]
−CO2
+[O]
−CO2
+[O]
−CO2
+[O]
EMP
1 CO2
2 CHO
3 CHOH
4 CHOH
5 CHOH
6 CH2-O-P
Extensive pentose cycle
Limited pentose cycle
22 C
33 C
32 C
44 C
55 C
66 C
2 C
3 C
C2-C3 cleavage
4 COOH
5 CHOH
6 CH3

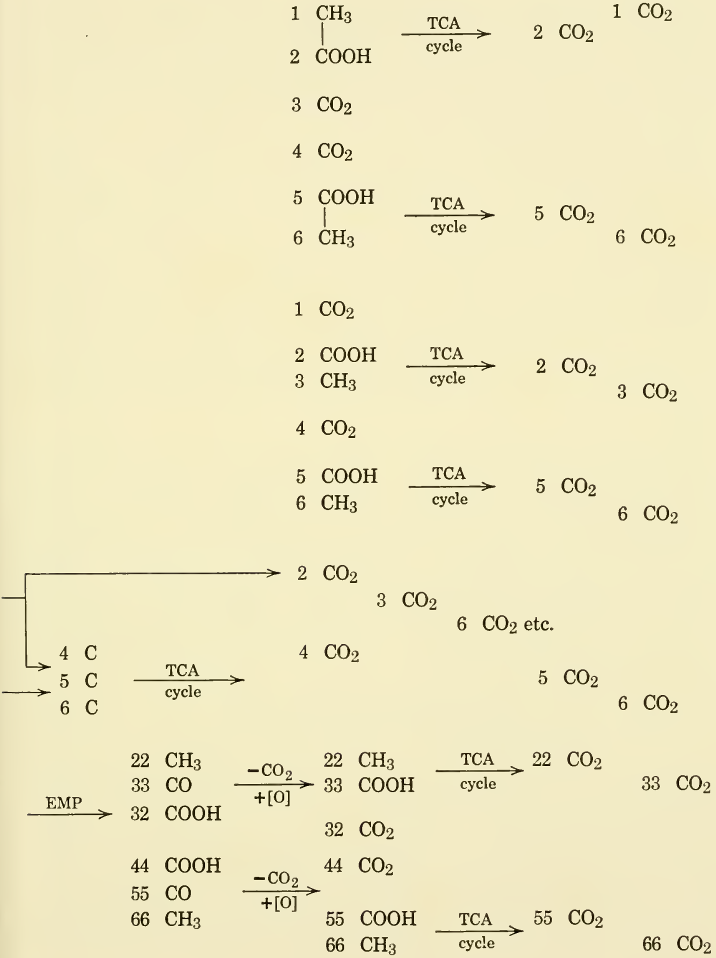


Fig. 2.5. Relative rates of CO_2 production from glucose via different pathways; identity of glucose carbons in other intermediates.

EMP = Glycolysis (Embden-Meyerhof-Parnas)
ED = Entner-Doudoroff
HMP = Hexose Monophosphate Pathway (phosphogluconate cleavage) (including pentose cycle)

since the ratio of $C^{14}O_2$ production, C_1/C_6, does not carry any quantitative significance unless the actual amounts of substrate glucose are known. Moreover, the evolution of CO_2 from carbons 3 and 4 (reflecting glycolysis) was not taken into account.

Major objections to the use of specific activities of $C^{14}O_2$ to measure pathway participation are two: (*a*) whenever more than one pathway is operative, as is usually true, regeneration of hexoses by the pentose cycle results in unpredictable dilution of the respiratory CO_2, from glucose carbon atoms that undergo glycolysis or other breakdown; (*b*) there is a dilution of all C^{14} atoms (5) by endogenous metabolites, which are oxidized and enter the total CO_2 pool.

The latter objection may also be raised against methods that employ measurement of the specific activity of such intermediates as pyruvate, lactate, or alanine. Weinhouse and co-workers (3) have met this by the ingenious device of using glucose-U-C^{14} in concurrent experiments as a reference standard, so that the dilution may be recognized and compensated. However, this method also assumes that there exists no drainage of pentose cycle intermediates for synthetic purposes, and it assumes further that pentose P does not re-form hexose P. The error caused by this last assumption will be small if the contribution that is made by the pentose cycle to total metabolism is small. Dawes and Holms (6–8) have assumed, correctly, I believe, that any regenerated hexose will be catabolized by *both* glycolysis and the pentose pathway; however, they have also assumed no drainage of pentose cycle intermediates for synthesis.

In a comprehensive, thoughtful analysis of this subject, Katz and Wood (9) have recognized the dilution problem caused by oxidation of regenerated endogenous metabolites, and have attempted to account for the recycling of carbon atoms that occurs in the pentose cycle; extensive calculations are offered to show how this recycling would influence the specific activities of respiratory CO_2 from carbons 1, 2, and 3 of glucose. However, although these workers assume that glucose-6-P and fructose-6-P are in complete equilibrium, their treatment of data does not seem to allow adequately for the possibility of diversion of hexose-P from the pentose cycle into glycolysis. Their calculations appear, in effect, to assume that carbon in the pentose cycle tends to be recycled until it is oxidized to CO_2. Moreover, no drainage of the cycle intermediates is envisaged for other cellular functions, and it is assumed that pentose cannot be formed by reversal of the cycle, that is, by reversal of the transaldolase-transketolase sequence. These points will be dealt with in greater detail in the discussion on *B. subtilis* oxidations, where the bulk of glucose catabolism is routed via glycolysis, and also under the topic of the role of the *reductive* pentose cycle in organisms.

RADIORESPIROMETRY

The third method has been called by us the radiorespirometric method. You might expect that we would favor it, since it was developed in our laboratories; but I believe it to possess certain attractive features, and since we are especially familiar with the method, I would like to explain it to you.

As developed by Dr. Wang and his colleagues, the radiorespirometric method does not stress specific activities; it measures instead the *yields* of $C^{14}O_2$ from various metabolites. The range of readily available compounds includes glucose 1-, 2-, 3-, 3,4-, 6-, and U-C^{14}; similarly labeled gluconate; pyruvate 1-, 2-, and 3-C^{14}; and acetate 1- or 2-C^{14}. These substrates represent all major sources of carbon in the currently recognized metabolic pathways.

The apparatus used is represented in the sketch in Fig. 2.6. As will be seen, it consists of a modified Warburg

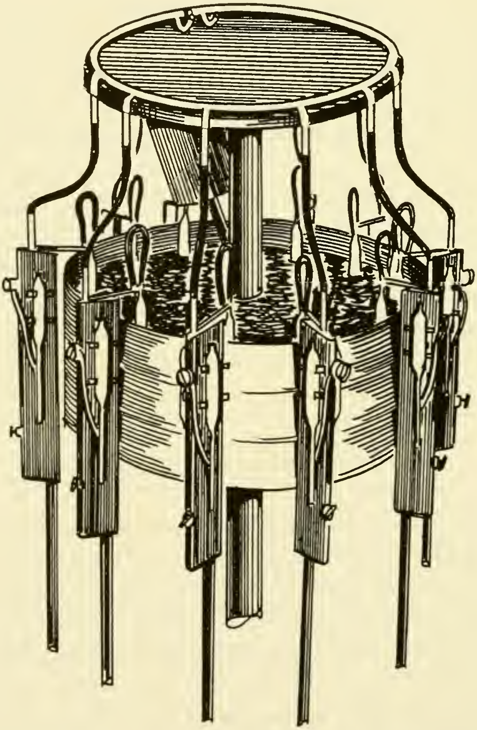


Fig. 2.6. The interval radiorespirometer. (Courtesy of Krishell Laboratories, Portland, Oregon.)

apparatus, in which the reaction flask and CO_2 receiver are constructed as shown in Fig. 2.7. It may be noted that the manometer of the usual apparatus has been exchanged for a system for trapping respiratory CO_2; by proper manipulation of the stopcock above the CO_2 trap (G in Fig. 2.7), shifts can be made to alternate receivers. (For operating instructions, see reference 11.) Shifts to new receivers permit analysis of the KOH solution holding the recovered CO_2, and thus the CO_2 obtained in a desired interval of time may be recorded; the apparatus becomes an *interval* radiorespirometer. Plots of the interval yields of $C^{14}O_2$ from the labeled substrate obtained automatically display the kinetics of the process. It will also be noted that several simultaneous experiments may be carried out; by using differently labeled substrates in each flask, a battery of experiments may be conducted that will give a simultaneous picture of the kinetics of oxidation of each of several carbon atoms of a given substrate. The uncontrollable complexities of metabolism that cause dilution of individual carbon atoms and spuriously influence specific activities may be ignored since the instrument permits a measure of the total yield of CO_2 from a given labeled position of a substrate molecule.

It is reasoned that oxidation of glucose exclusively at carbon 1 is indicative of phosphogluconate cleavage. Any entrance of carbon 1 into CO_2 via the Embden-Meyerhof route followed by the TCA cycle should also be reflected in equal C_6 oxidation; so the difference between the C_1 and C_6 yield in respiratory CO_2 should give a measure of the extent of phosphogluconate cleavage. The per cent of the *total* metabolism of glucose that this process supports, on the other hand, will be chiefly influenced by the appearance of carbons 3 and 4 in CO_2 (indicative of glycolysis). The

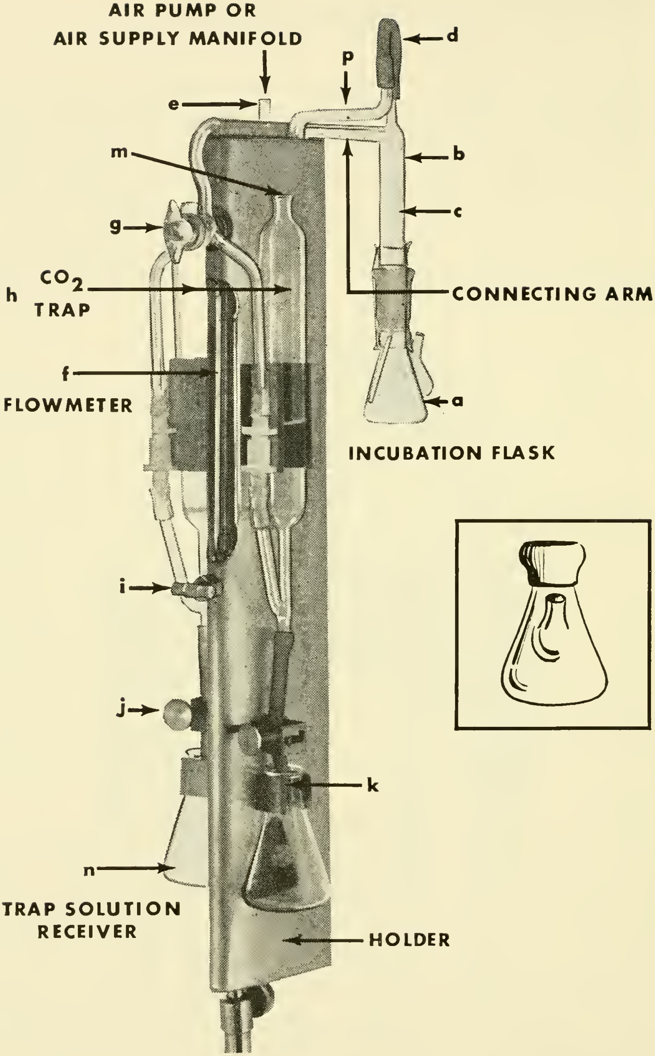


Fig. 2.7. Details of reaction flask and CO_2 traps in interval radiorespirometer. *a,* incubation flask and side arm; *b,* head for connection

calculation of the fraction of the total metabolism that travels by the pentose pathway (with and without recycling) is measured as

$$G_p = \frac{G_1{}^1 - G_6{}^1}{G_T}$$

where G_p = fraction of glucose participating in phosphogluconate decarboxylation

$G_1{}^1$, $G_6{}^1$ = total activity in respiratory CO_2 from cells utilizing equal amounts of C-1 or C-6 labeled glucose

G_T = total activity of the administered substrate (always taken as 100%, or unity)

The fraction of glucose catabolized by glycolysis, G_e (Embden-Meyerhof-Parnas pathway) is

$$G_e = 1 - G_p$$

This assumes that the Entner-Doudoroff pathway does not simultaneously operate. In our experience with various organisms, this assumption seems generally safe. When the Entner-Doudoroff pathway is present, glycolysis is seen generally to have subsided, and the recognition of Entner-Doudoroff activity (separately from the pentose cycle) is made by noting the difference between the yield of CO_2 from carbons 1 and 4 of glucose, since exclusive Entner-Doudoroff

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of incubation flask; *c* and *p*, aeration tubing (glass) with lower end protruding into incubation flask; *d*, rubber tubing; *e*, air inlet; *f*, flowmeter; *g*, three-way stopcock; *h*, CO_2 trap; *i*, needle valve, to regulate gas flow; *j*, tension clamp for release of trap solution; *k*, clamp for holding trap solution receiver; *n*, trap solution receiver. (Courtesy of Krishell Laboratories, Portland, Oregon.)

activity would show equal yields of CO_2 from carbons 1 and 4 of glucose.

$$G_p = \frac{G_1{}^1 - G_4{}^1}{G_T}$$

G_{ed} (Entner-Doudoroff) is taken as the remainder

The types of CO_2 production curves obtained vary from organism to organism; four of these are reproduced here to show the characteristic differences that exist.

1. In bakers' yeast, Fig. 2.8*A* shows that the greatest evolution of CO_2 is from carbons 3 and 4, characteristic of glycolysis. (The general availability of this species of glucose dictates its routine use over glucose-3-C^{14}; it is assumed here that the yield in CO_2 is equal from both carbon atoms.) $C^{14}O_2$ production from C-1, although higher at first than from C-6, is much lower than from C-3,4. The curve for interval recovery of CO_2 from C-3,4 reaches a maximum at about 2 hours, then drops to a minimum at 4 hours; the latter time coincides with complete removal of the administered glucose from the medium. This time is defined as 1 relative time unit (1 RTU) and is used to make comparisons more relative among different organisms (*Streptomyces griseus,* for example, exhibited a much slower metabolism under the experimental conditions used than did bakers' yeast; 1 RTU was about 10 hours). After 1 RTU the accumulated radioactivity in the cells and medium undergoes depletion; yields of $C^{14}O_2$ from carbons 2 and 6 increase in that order, as would be expected from oxidation of acetate via the TCA cycle.

2. The second type is exemplified by *Zymomonas mobilis;* the $C^{14}O_2$ yields agree with the contention that the

Entner-Doudoroff pathway predominates, since the amounts are essentially equal from C-1 and C-4 of glucose. This is revealed in Fig. 2.8*B*, since there is reported to be no Krebs TCA cycle in this organism. There is therefore no second phase after 1 RTU in which $C^{14}O_2$ is formed from carbons 2 or 6, as in yeast.

3. Phosphogluconate cleavage, followed by the complete pentose cycle, is seen in *A. suboxydans* (Fig. 2.8*C*). In this curve, both interval and total yields are greatest from glucose C-1, >C-2, >C-3,4, >C-6. This is precisely in accordance with expectations. The greater yield of C-1 over C-2 in the respiratory CO_2 suggests that pentose cycle activity is not as great as phosphogluconate cleavage; some C-2 is evidently utilized in pentose that undergoes assimilation. The Entner-Doudoroff route is ruled out because of the high yield of CO_2 from glucose C-2 (higher than from C-4). If *A. suboxydans* utilized the Entner-Doudoroff pathway, and then diverted the triose formed (from carbons 4, 5, and 6) into hexose phosphate via aldolase condensation and degraded the latter via the pentose cycle, the $C^{14}O_2$ would be fairly rich from carbon 6. C-2, however, would be retained as acetate, since pyruvate is converted nearly quantitatively to acetate in this organism (12).

With these alternate pathways eliminated, the contribution of the pentose cycle to total glucose dissimilation in *A. suboxydans* can be estimated quantitatively with the use of the cumulative recovery data of Table 2.1. Thus, in an oxygen atmosphere, at 1 RTU, conversion of glucose to pentose phosphate = C-1 recovery = 72%.[2] Pentose phosphate undergoing cycling = C-2 recovery = 63% of glucose administered (hence, $(63 \times 100)/72 = 88\%$ of pentose formed).

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[2] 2-Ketogluconate accounted for all (28%) of the glucose that was not converted to pentose in this experiment.

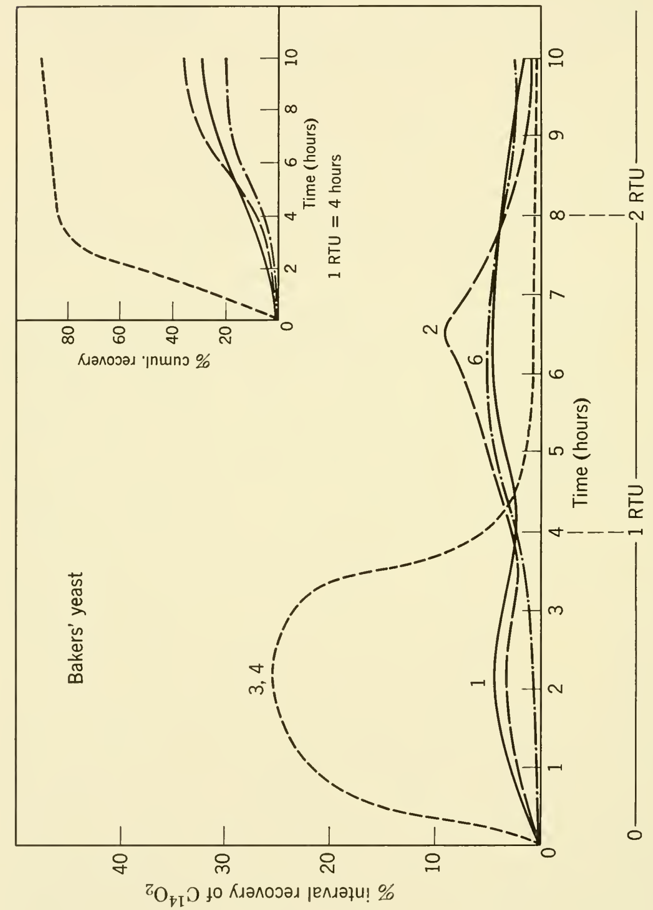
Bakers' yeast
% interval recovery of $C^{14}O_2$
0
10
20
30
40
Time (hours)
0
1
2
3
4
5
6
7
8
9
10
3, 4
1
2
6
% cumul. recovery
20
40
60
80
Time (hours)
0
2
4
6
8
10
1 RTU = 4 hours
0
1 RTU
2 RTU

Fig. 2.8 Part (*A*).

Fig. 2.8. Time-course plots of radiochemical recoveries in CO_2 from metabolizing specifically labeled glucose.

(*A*) Bakers' yeast: 1 RTU = 4 hours.
(*B*) *Zymomonas mobilis* (formerly called *Pseudomonas lindneri*): 1 RTU = 4 hours.
(*C*) *A. suboxydans:* 1 RTU = 4 hours.
(*D*) *B. subtilis:* 1 RTU = 4.5 hours.

In all systems, the following legends are employed: ————, glucose-1-C^{14}; — — — — —, glucose-2-C^{14}; - - - - - -, glucose-3,4-C^{14}; ·······, glucose-4-C^{14}; and —·—·—·—, glucose-6-C^{14}. Inserts: cumulative recoveries of $C^{14}O_2$.

Since oxidation of C-6, -5, or -4 required recycling, we can assume that the maximal yield of C-4 in CO_2 will be approximately 88% of C-6, = (33/100) × 0.88 = 29%. Also, since C-3, 4 "average" recovery = 46%, recovery of C-3 + C-4 = 92%. Therefore, minimal recovery of C-3 = (92 − 29)/100 = 63%.

This calculated value for recovery of C-3 in the respiratory CO_2 agrees well with the observed value for C-2 recovery from glucose (63%; Table 2.1) and thus confirms the pentose cycle as the only significant pathway for *complete* oxidation of glucose in the organism concerned under the conditions which prevailed.

This calculation, although different from that of Katz and Wood (9) nevertheless takes into account much of the complexity of recycling of F-6-P. In an organism that relies on the pentose cycle as extensively for oxidation of glucose, one would expect that in resting cells the combustion of glucose carbon atoms 2 through 6 should approach equal magnitude with carbon 1, although they may do so more slowly. The fact that they do not, even at the end of the

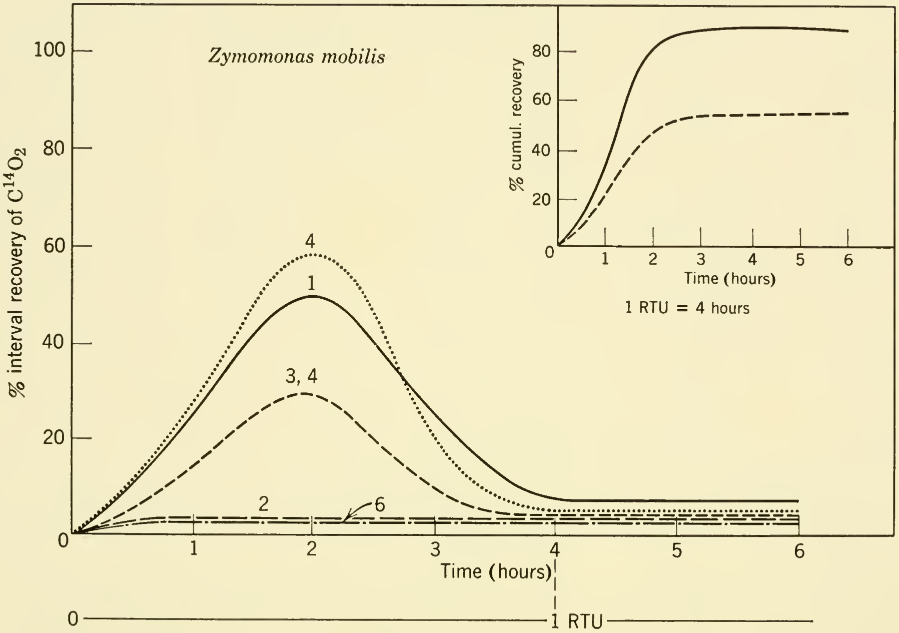

Fig. 2.8 *(continued)*. Part *(B)*.

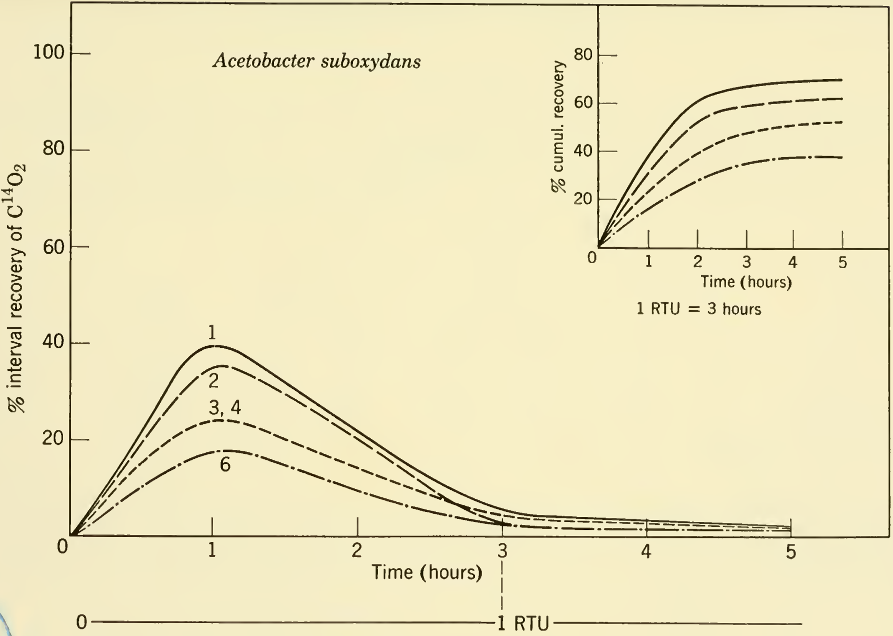

Fig. 2.8 *(continued)*. Part *(C)*.

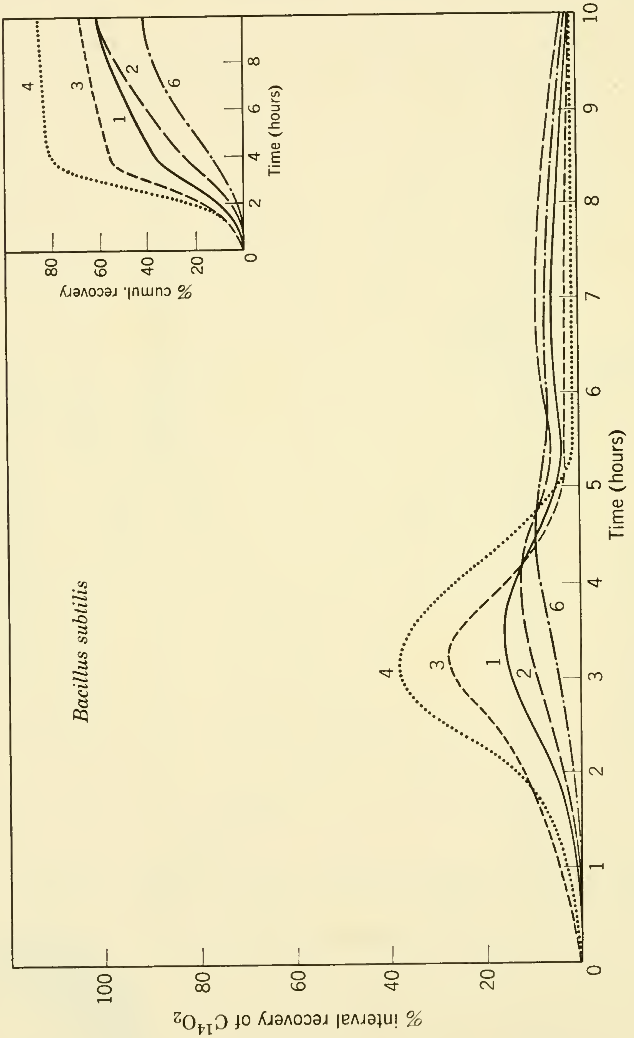

Fig. 2.8 *(continued)*. Part *(D)*.

TABLE 2.1

Dissimilation of Glucose by Resting Cells of *Acetobacter suboxydans*

		Per Cent Recovery of C^{14}							
		CO_2		Cells		Medium		Total	
Substrate	CPM Added	Air *	Oxygen *	Air	Oxygen	Air	Oxygen	Air	Oxygen
Glucose-1-C^{14}	70,000	80	72	2	0	20	27	102	99
Glucose-2-C^{14}	70,000	65	63	4	2	34	36	103	101
Glucose-3,4-C^{14}	70,000	51	46	6	3	44	51	101	100
Glucose-6-C^{14}	60,000	33	33	5	3	61	65	99	101
Glucose-C^{14}, uniformly labeled	70,000	55		7		36		98	

* Air represents air atmosphere and 1 mg. of cells, dry weight, per milliliter of medium; oxygen represents oxygen atmosphere at the same cell concentrations. The time was 5 hours. Each flask contained 75 μmoles of glucose and 500 μmoles of phosphate buffer, pH 6.0. The volume was 10 ml. and the temperature 30°.

time-course experiment, strongly suggests drainage of carbon skeletons from the cycle.

4. The fourth example is given by *B. subtilis,* which appears to utilize a combination of pathways, much as yeast does. It is reproduced here because (*a*) the glycolysis pattern is less pronounced than in yeast, and (*b*) the data permit a testing of one of the main implications of the paper of Katz and Wood, namely that carbon atoms traversing the pentose cycle seemingly do so until oxidized, without escape of triose units.

One can readily recognize two distinct phases of glucose oxidation in *B. subtilis* (Fig. 2.8*D*), as in yeast, with the division occurring approximately at 1 RTU. At first, glycolysis is clearly indicated by the high recovery of glucose-C-3 and -4 in CO_2. Meanwhile, the presence of an alternate pathway is recognized, since the yields of $C^{14}O_2$ from these carbon atoms are not equal, nor are they equal between C-1 and C-6. The excess yield of C-1 over C-6 points to phosphogluconate cleavage as the alternate route involved. The fact that C-2 oxidation was less than C-1 again suggests that some pentose phosphate was assimilated —a conclusion to be expected since some cell proliferation took place in this experiment.

After exhaustion of the administered glucose in Fig. 2.8*D* there is a resumption of oxidation of carbons 2 and 6, and to some extent carbon 1. The order of release of these carbons into CO_2 (C-2 $\geqq$ C-6 > C-1 > C-3,4) reflects the operation of the TCA cycle.

The usefulness of the radiorespirometric experiments is revealed from the experiments in Fig. 2.9 on the utilization of gluconate by *B. subtilis.* Gluconate presumably cannot be converted directly back to glucose; its oxidation would seem to be obligatory by the pentose cycle, at least as far

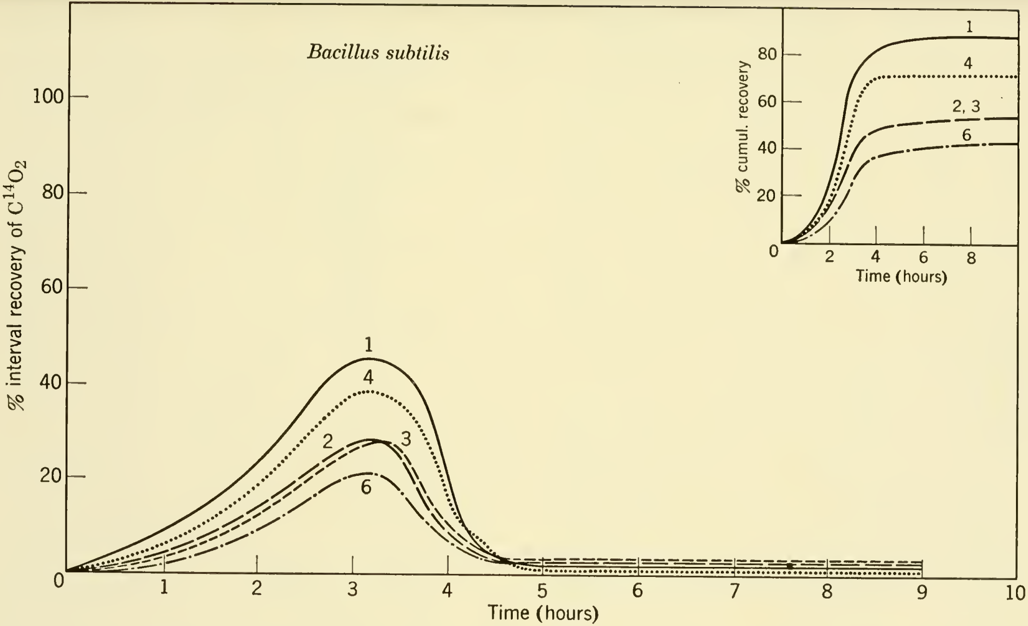

Fig. 2.9. Time-course plots of radiochemical recoveries of gluconate in $C^{14}O_2$. Organism: *B. subtilis.* Legend: ————, glucose-1-C^{14}; — — — — —, glucose-2-C^{14}; - - - - - -, glucose-3,4-C^{14}; ·······, glucose-4-C^{14}; and —·—·—·—, glucose-6-C^{14}. Inset: cumulative recoveries of $C^{14}O_2$.

as the formation of triose phosphate or fructose-6-phosphate. Now the disposition of these species is crucial: will the F-6-P re-enter the pentose cycle as seems obligatory from the paper by Katz and Wood, or will the triose be subjected in large part to degradation via the Krebs cycle, as indicated for this organism in the foregoing paragraphs?

The radiorespirometric patterns in Fig. 2.9 and Table 2.2 provide the following information: (*a*) gluconate is readily broken down, presumably after initial phosphorylation; (*b*) the route employed is not the Entner-Doudoroff pathway, either alone or in combination with the pentose cycle. In the Entner-Doudoroff pathway, there should be metabolic equivalence between C-1 and C-4, C-2 and C-5, or C-3 and C-6. This was not found. Instead, gluconate appears to be metabolized via a sequence (Fig. 2.10):

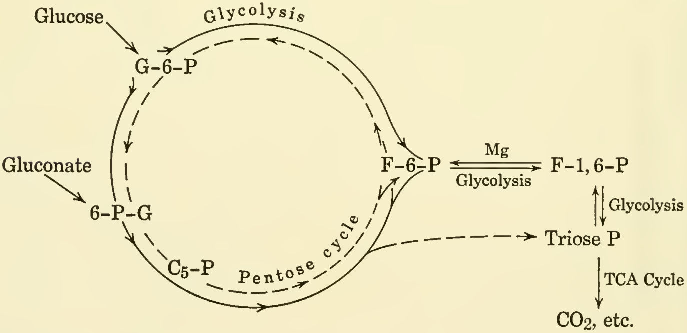

Fig. 2.10. Simultaneous operation of glycolysis and the pentose cycle, and their effect on metabolism of administered glucose and gluconate. Both circular loops represent pentose cycle operation: the outer loop for the degradation of gluconate and a part of glucose; the inner, for the portion of both glucose and gluconate metabolism that escapes glycolysis after the first formation of triose phosphate, i.e., the portion that recycles.

$$\text{Gluconate-6-P} \xrightarrow{\text{pentose cycle}} \text{fructose-6-P}$$

$$\begin{tikzcd}
 & \text{F-1, 6-diP} \arrow[r] & \text{glycolysis} + \text{TCA cycle} \\
\text{Fructose-6-P} \arrow[ur, "\text{major}"] \arrow[dr, "\text{minor}"'] & & \\
 & \text{G-6-P} \arrow[r] & \text{pentose cycle}
\end{tikzcd}$$

This conclusion is drawn from the consideration that the fructose-6-P derived from the labeled gluconate should bear the following labeling patterns (1) (see also Fig. 2.2):

$$\begin{array}{ccccccl}
 & 1\ C & & 1\ CO_2 & & & \\
 & & & + & & & \\
 & 2\ C & & 2\ C & & 2\ 2\ 2\ 2 & \\
 & & & & & 3\ 3\ 3\ 3 & \\
 & 3\ C & \rightarrow 6 & 3\ C & \rightarrow & 2\ 2\ 3\ 3 & +\ C\ 4\ 4 \\
6 & & & & & 4\ 4\ 4\ 4 & \ \ \ C\ 5\ 5 \\
 & 4\ C & & 4\ C & & 5\ 5\ 5\ 5 & \ \ \ C\ 6\ 6 \\
 & & & & & 6\ 6\ 6\ 6 & \\
 & 5\ C & & 5\ C & & & \\
 & 6\ C & & 6\ C & & \multicolumn{2}{l}{4\ \text{F-6-P} + 2\ \text{triose-P}}
\end{array}$$

Further catabolism of F-6-P may be predicted as follows. Let us consider the fate of gluconate-C-2:

In Table 2.2, data are provided on the total recoveries of $C^{14}O_2$ from individual carbon atoms of glucose and gluconate. For gluconate, the maximum fraction decarboxylated is 88%. This represents the maximum amount of pentose phosphate that could have traversed the cycle, and the probable actual amount that did so.

According to the scheme just presented, the re-formed fructose-P (from gluconate) will have the original C-2 from gluconate in carbons A and C (1 and 3). Sixty-seven per cent of this will be in carbon A, 33% in carbon C.

TABLE 2.2

Yields of Individual Atoms from Glucose and Gluconate: *B. subtilis* Oxidations

(Synthetic Medium)

	$C^{14}O_2$ Recovery			
Substrate	CO_2	Cells	Medium	Total
Gluconate-1	88%	1%	11%	100%
Gluconate-2	54%	22%	18%	94%
Gluconate-3	54%	31%	17%	102%
Gluconate-3,4	63%	19%	15%	97%
Gluconate-4	72%	7%	13%	92% (calc.)
Gluconate-6	44%	36%	18%	98%
Glucose-1	60%	22%	13%	95%
Glucose-2	60%	25%	16%	101%
Glucose-3	67%	19%	16%	102%
Glucose-3,4	77%	15%	12%	104%
Glucose-4	87%	11%	8%	106% (calc.)
Glucose-6	41%	23%	41%	105%

If we now assume that re-formed *fructose* behaves like administered *glucose,* Table 2.3 tells us that carbons A and C of glucose will be recovered in CO_2 to the extent of 60 and 67%, respectively. Multiplying

$$88\% \times 67\% \times 60\% = 35\%$$

and multiplying

$$88\% \times 33\% \times 67\% = \underline{20\%}$$

55% calculated recovery of $C^{14}O_2$
from C-2 of gluconate

This agrees well with the observed recovery of 54% from C-2 of gluconate (Table 2.3). Formation of $C^{14}O_2$ from

TABLE 2.3

Data Analysis—*B. subtilis* Synthetic Medium

		June 24, 1960	Fraction of Gluconate De-CO_2-ed A	Fraction of Relocated Gluconate-C in Glucose △ B	Recovery of Glucose-C in CO_2 at 10 Hours C	Calculated $C^{14}O_2$ Recovery (Fraction) A·B·C	Calculated $C^{14}O_2$ Recovery (Total) %	Observed $C^{14}O_2$ Recovery %
		Gluconate-2	0.88	0.67 in C-A	0.60	0.35	55%	54%
			0.88	0.33 in C-C	0.67	0.20		
A	CHO	Gluconate-3	0.88	0.67 in C-B	0.60	0.35	55%	54%
B	C		0.88	0.33 in C-C	0.67	0.20		
C	C	Gluconate-4 *	0.88	1 in C-D	0.87	0.77	77%	72%
D	C	Gluconate-4 †	0.88	0.83 in C-D	0.87	0.64	74%	72%
E	C		0.88	0.17 in C-C	0.67	0.10		
F	CH_2OH	Gluconate-6 *	0.88	1 in C-F	0.41	0.36	36%	44%
		Gluconate-6 †	0.88	0.83 in C-F	0.41	0.30	39%	44%
			0.88	0.17 in C-A	0.60	0.09		

* Assuming no triose recombination.

† Assuming triose recombination.

△ According to scheme by Beevers (Fig. 2.2).

After Cheldelin et al. (12).

other positions in gluconate also agrees fairly well with predictions. Nearly identical results were obtained in a repeat experiment, not shown here, performed six weeks later.

It would appear from the above paragraph that fructose exists in a general pool in this organism, and that it is treated the same whether it is administered as substrate or re-formed from the pentose cycle; it is apparently unnecessary to make so extensive allowance for recycling as Katz and Wood have done. Instead of a perfect equilibrium existing among fructose-1, 6-diP, glycolysis, and the pentose cycle, there appears instead to be a steady state that, in *B. subtilis* at least, is shifted toward glycolysis and the TCA cycle. These data support the assumption made by Dawes and Holms (13) in *Sarcina lutea,* that re-formed fructose will be degraded both by glycolysis and the pentose cycle. It would appear likely that this condition may obtain in animal systems as well; work is being planned in our laboratories to test this assumption further.

QUANTITATIVE EXPRESSION OF PATHWAY PARTICIPATION

The reader may now inquire whether so simple a calculation as we have previously employed (11), can really represent the complex events that take place in organisms,

$$\mathrm{G}_p = \frac{\mathrm{G}_1 - \mathrm{G}_6}{\mathrm{G}_T} \qquad \text{(footnote 3)} \qquad (1)$$

in spite of the seeming reinforcement given by the *B. subtilis* experiment quoted. The fact is that the *B. subtilis*

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[3] In the original report (11), G, and G_6 were written as G_1' and G_6'.

experiment merely equates fructose regenerated in the pentose cycle with the glucose administered as substrate. The equation used previously is indeed too simple; data obtained through its use are somewhat in error, as follows:

When an organism degrades glucose by both the glycolysis–Krebs cycle and the pentose cycle routes, some triose phosphate will be formed by either pathway. Glucose carbons 1 and 6 will appear in triose in equal amounts by glycolysis, but the triose from the pentose cycle will contain only carbons 4, 5, and 6 (carbon 1 will have been converted to CO_2). The total triose pool will therefore be enriched with carbon 6, and carbon 6 yields in CO_2 will be too high.

This means that corrections should be provided which enlarge the differences between G_1 and G_6, specifically by reducing G_6; in other words, the reported contribution of phosphogluconate cleavage, as calculated by equation 1, is spuriously low. Such refinements have been made (12, 14), and it is believed that the newer equations will give a more nearly correct evaluation of simultaneously participating pathways. Briefly, where glycolysis and phosphogluconate cleavage occur together, Wang (12, 14) has developed the following expression:

$$G_p = \frac{G_1 - (G_6 - A_6 G_p)}{G_T - G_T'} \qquad (2)$$

where G_T = total radioactivity of each administered substrate (always taken as 100%, or unity)

G_T' = fraction of the labeled substrate engaged in anabolism

G_1, G_6 = per cent total yields of $C^{14}O_2$ from systems metabolizing equal amounts of glucose labeled in these positions

A_6 = per cent total $C^{14}O_2$ yields from systems metabolizing equal amounts of gluconate labeled in these positions

G_p = fraction of the administered glucose catabolized via phosphogluconate cleavage

In equation 2 a correction factor has been introduced into the term G_6 (equation 1). The correction arises from the understanding that the term G_6 in reality represents a total conversion of C-6 of glucose to CO_2 via both glycolysis and the pentose cycle pathways, whereas the derivation of equation 1 calls for the consideration of CO_2 production from C-6 of glucose via the glycolysis exclusively. The CO_2 production from C-6 of glucose via the pentose cycle reactions can be represented as $A_6(G_p)$ since G_p represents the fraction of glucose routed into the pentose cycle and A_6 represents the CO_2 yield from C-6 of glucose by way of phosphogluconate. Consequently, the term $(G_6\text{-}A_6G_p)$ represents the net production of CO_2 from C-6 of glucose via the glycolysis–Krebs cycle pathway which is theoretically identical to the production of CO_2 from C-1 of glucose via the same metabolic route.

When equation 2 is used in place of equation 1 to calculate the pathway participation in *B. subtilis,* the previous value of 26% for phosphogluconate decarboxylation rises to 41%. Similar refinements (including a determination of gluconate conversion to CO_2) are in progress with several other organisms. Table 2.4 lists several organisms in which the extent of participating pathways is reasonably well known, although several may be revised when adequate values for gluconate oxidation are available.

Knowledge of catabolism of glucose via catabolic pathways in animals is in a less satisfactory state, although in

TABLE 2.4

Estimation of Catabolic Pathways of Glucose
Pathway Estimation

Microorganism	Glycolysis	HMP	ED	Method	Reference
Saccharomyces cerevisiae	88	12		Radiorespirometry	11
	83–100 *	0–17 *		Specific activity of intermediates	3
Candida utilis	66–81	19–34		Specific activity of intermediates	3
Streptomyces griseus	97	3		Radiorespirometry	11
Penicillium chrysogenum	77	23		Radiorespirometry	11
Penicillium digitatum	77–83	17–23		Radiorespirometry	11
Fusarium lini	83	17		Specific activity of $C^{14}O_2$	16
Escherichia coli	72	28		Radiorespirometry	11
Sarcina lutea	70	30		Radiochemical yield of intermediates	6
Bacillus subtilis	74	26		Radiorespirometry; equation 1	
	59	41		Radiorespirometry; equation 2 †	
Pseudomonas reptilivora		28	72	Radiorespirometry	15
Pseudomonas aeruginosa		29	71	Radiorespirometry	15
Acetobacter suboxydans		100		Radiorespirometry	4
Zymomonas mobilis			100	Radiorespirometry	15
Pseudomonas saccharophila			100	Radiorespirometry	15

* Experimental conditions varied from growing to depleted resting cells.
† Equation 2 is used with the assumption that $GT' = 0$.

general it may be stated that it appears that glucose is catabolized mainly by way of glycolysis and the Krebs cycle; phosphogluconate cleavage may participate to a minor, variable extent (17). Qualitative experiments have been reported by Bloom (18) on a variety of rat tissues; by others on rats (19–23), blood cells (24), insects (25), cow's mammary gland (26), retina (27), and carp (28). Interesting side reactions have been uncovered; the occurrence of the pentose cycle as well as the extent of recycling of pentose phosphate in cows were reported by Wood (29). By studying the isotopic distribution patterns in amino acids, glycerol, and glucose isolated from milk derived from cows fed with specifically labeled substrate, it was possible to detect transaldolase exchange, and to enable the author to examine the possible effect of side reactions upon the estimate of glucose metabolic pathways.

Other reports in the literature permit speculation on the probable role of the pentose cycle in over-all metabolism. These speculations will be reserved for the next lecture, where a more extensive discussion of this cycle will be made.

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CHAPTER

3

CARBOHYDRATE METABOLIC PATHWAYS: GENERAL CONSIDERATIONS

In the two previous chapters we have discussed the peculiar metabolic events that occur in *Acetobacter suboxydans,* and how some of the major carbohydrate degradation routes are measured in respiring organisms. In this chapter I should like to strike a somewhat more general note, to discuss some of the processes with which most students of carbohydrate metabolism are already familiar, but in the hope that our fresh discussion may divert a few rays of light in a new manner upon the complexities of total metabolism in a whole tissue or organism.

Let us begin this discussion by again referring to the Krebs tricarboxylic acid cycle. This mechanism deserves special recognition because its discovery, by Krebs and Johnson almost a quarter century ago (1), ranks as one of the major landmarks in all biochemistry. Its primary importance to students of carbohydrate metabolism can best be emphasized by the simple diagram of the Krebs cycle shown in Fig. 3.1. This sequence shows how compound A (pyruvate) can be converted to B (acetyl CoA) which, in turn, combines with a molecule of C (oxalacetate) to produce D (citric acid) and continue stepwise to the re-formation of compound C.

Viewed in this light the Krebs cycle is revealed for what it really is—a catalytic cycle. As has been pointed out previously (2), the removal of a few molecules of any of the intermediates pictured here *for synthesis* stops the cycle, unless there is some provision for the independent formation of di- or tricarboxylic acids. Thus we find in most organisms a functioning malic enzyme, or oxalacetic decarboxylase, or malate synthetase system; if one or more of these is operating there is an additional source of Krebs cycle intermediates, so that removal of one or more of the intermediates for synthesis of amino acids, for example, may be possible. Without such independent mechanisms, the Krebs cycle as we usually picture it could not operate, and energy metabolism therefrom would soon also grind to a halt.

Another reason for making initial mention of the citric acid cycle is that it helps to emphasize the importance that this mechanism has held, not only as a pathway of metabolism, but also perhaps even more importantly as a

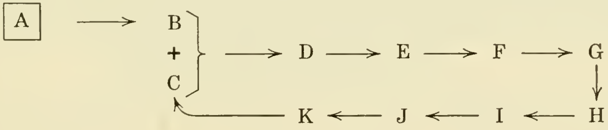

Fig. 3.1 The Krebs tricarboxylic acid cycle—a schematic diagram.

conceptual contrivance that has made possible many extensions of knowledge about metabolism in general. Because the individual steps in the pathway have been verified, the viewpoint is sometimes diminished that the cycle is not only a proven piece of metabolic machinery, but also may be a powerful psychological tool in helping to chart new ground.

THE MITOCHONDRION AND THE CITRIC ACID CYCLE

The ubiquity of the Krebs cycle was given deepened appreciation during the early 1940's, with the discovery by

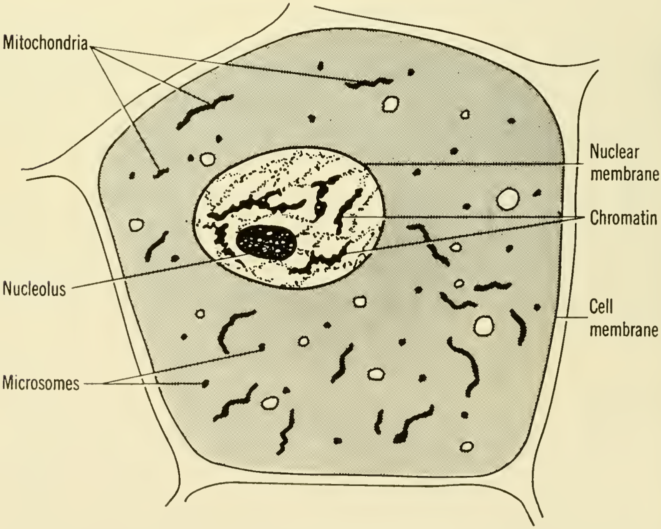

Fig. 3.2. Diagram of a generalized cell.

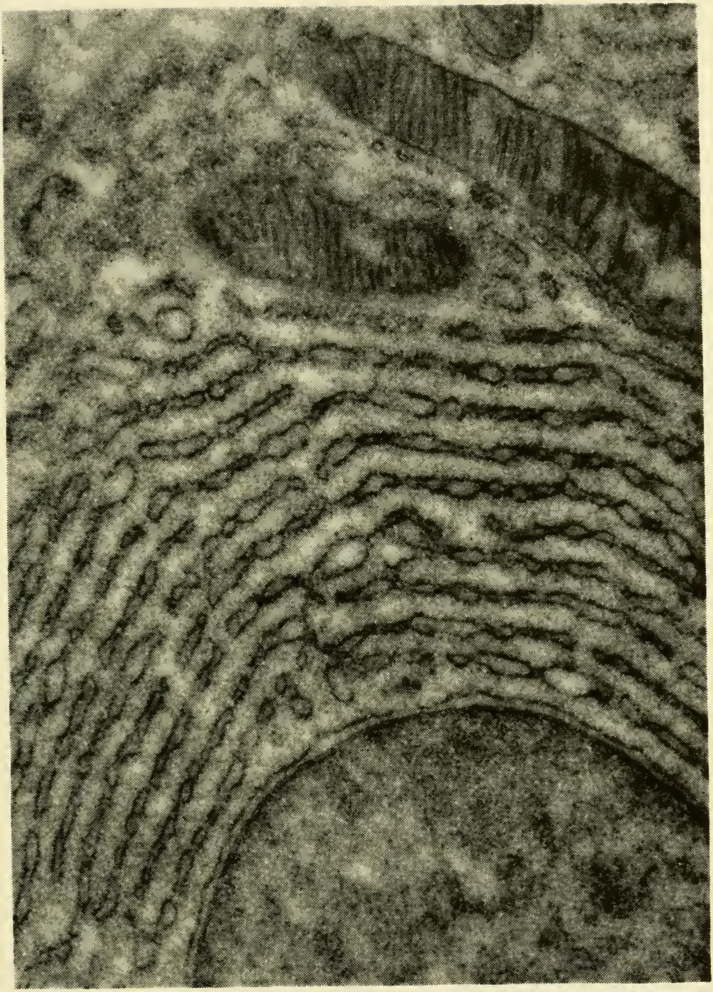

Fig. 3.3. Mitochondria and microsomes in the liver. Ultrathin section of mitochondria from a rat liver cell. Mitochondria showing internal cristae. In the surrounding cytoplasm canaliculi of the endoplasmic reticulum with dense granular material in the outer surface are seen. Magnification = 25,500×. [After Palade (5).]

Claude (3, 4) and others, of the close association between the group of enzymes necessary for the process, and their presence in mitochondria.

Consider for a moment the inclusions, or subcellular particles, that may be observed in a typical cell. A glance at the diagram of a generalized cell in Fig. 3.2 will reveal the nucleus, the large moon-shaped object in the upper center; the mitochondria, or "large granules," as these are called, which are smaller, yet easily visible under an ordinary high-power microscope; and the microsomes, or endoplasmic reticula, which resemble small strings of spaghetti, and which can be viewed in some detail in an electron micrograph. Other inclusions may be seen at times, such as food particles; but the foregoing are the principal ones. Palade's work, one of the plates of which is reproduced in Fig. 3.3 (5), has contributed much to our knowledge in this area.

Examples of the association between Krebs cycle enzymes and mitochondrial activity were to be found easily. With

TABLE 3.1

Size, Sedimentation Time, Gross Composition, and Succinoxidase in Liver Cellular Fractions

		Sedimentation		Fraction (per cent) of Total		
Fraction	Diameter, μ	Time, minutes	Acceleration	N	RNA	Succinoxidase
Nuclei	50–100	10	600g	15	10	10
Mitochondria	1–3	20	24,000g	30	25	90
Microsomes	0.06–0.15	120	41,000g	15	>35	0
Soluble	—	—	—	40	25	0

After Lehninger (6).

advances in refrigerated centrifugation, it was discovered that the cell fractions discussed could be separated readily: the nuclei and cell debris at centrifugal speeds of 1000*g* or less; mitochondria at 5000–20,000*g*; and microsomes at 25,000–50,000*g*. When this was done, Lehninger found (Table 3.1) (6) that whereas the nuclei, microsomes, and

TABLE 3.2

Complete Oxidation of Members of the Citric Acid Cycle

Substrate	Amount Added, μmoles	Oxygen Consumption: Observed, μatoms	Oxygen Consumption: Theory for Complete Oxidation, μatoms
α-Ketoglutarate	5	38.1	40.0
Malate	5	30.7	30.0
Citrate	5	47.1	45.0
Isocitrate	5	45.3	45.0
cis-Aconitate	5	48.4	45.0
Succinate	5	34.3	35.0
Fumarate	5	34.0	34.0
Pyruvate	5	23.9	25.0
Oxalacetate	2.5	12.2	12.5

	Substrate Oxidized, μmoles	Carbon Dioxide: μmoles	Carbon Dioxide: μmoles
α-Ketoglutarate	4.37	21.3	21.9
cis-Aconitate	5.00	27.9	30.0
Isocitrate	3.79	21.7	22.9
Succinate	3.77	15.1	17.2
Fumarate	4.73	16.4	18.9

Each flask contained 1.5 ml. of kidney mitochondria, 3 μmoles ATP, 4 μmoles $MgSO_4$, 50 μmoles phosphate buffer, pH 7.2, and substrate as indicated.

After Green et al. (7).

soluble enzymes had virtually no power to oxidize Krebs cycle intermediates (succinoxidase activity), the mitochondria by contrast showed a great ability to do so. In Table 3.2, Green et al. (7) have assembled all of the Krebs cycle intermediates and have shown that kidney mitochondria can oxidize these substrates completely, or, as shown in the lower half of the table, can produce CO_2 from these oxidations in theoretical yields. If one assumes the presence of enzymes that will produce pyruvate from glucose in an organism, it is clear that the oxidation of carbohydrates, the first great class of foodstuffs, can be explained by the activity of mitochondria; extramitochondrial Krebs cycle oxidations are rare in most organisms.

The mitochondria may be shown to be potent fatty acid oxidizers also. As Table 3.3 shows, caproic acid is oxidized by rabbit liver mitochondria, in amounts equivalent

TABLE 3.3

Oxidation of Caproic Acid to Acetoacetic Acid, Carbon Dioxide, and Water in Rabbit Liver Mitochondria

Caproate, μmoles	Theoretical Oxygen Requirement, μatoms	Oxygen Consumed, μatoms	AcAcOH Produced, μmoles	Caproate Oxidized Calculated from Oxygen Consumed, μmoles	O_2/CO_2 (theory = 4)
15	120	105	13.2	13.1	3.97
15	120	101	13.1	12.7	3.85
15	120	96	13.1	12.0	3.68
30	240	92	12.1	11.5	3.80
30	240	142	17.9	17.8	3.93
30	240	163	21.9	20.4	3.74

After Cheldelin et al. (8).

to the acetoacetate produced (acetoacetate is generally recognized as a by-product of fatty acid oxidation in the liver). Moreover, it may be seen that *all* of the oxidized fatty acid follows this route. The mechanism of fatty acid oxidation is depicted in Fig. 3.4, and it will be observed that there is continuing production of a two-carbon moiety (acetyl CoA) which, as discussed above, is burned completely to CO_2, H_2O, and energy via the Krebs cycle. All of the enzymes connected with this β-oxidation of fatty acids are in the mitochondria, and although a few examples may be found throughout the literature of extramitochondrial oxidation of fatty acids, e.g., in peanut microsomes (9), the great bulk of fatty acid oxidation appears to be of mitochondrial origin. The oxidation of the second major class of foodstuffs, viz., fats, thus appears to fall into line with carbo-

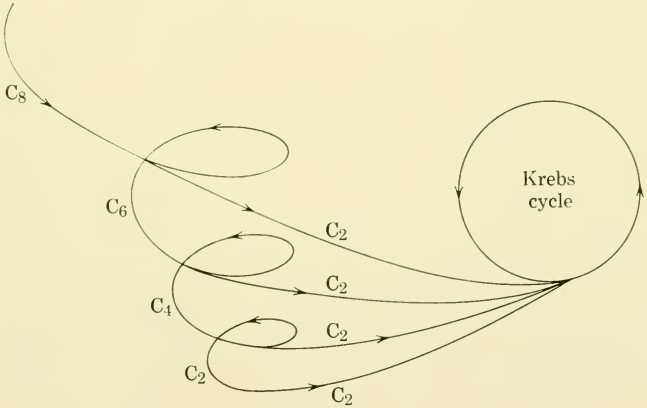

Fig. 3.4. Mechanism of fatty acid oxidation: the fatty acid spiral. Example: octanoic acid.

hydrate oxidation, so far as intracellular location of the enzymes is concerned.

The oxidation of the third major class of foodstuffs, namely the amino acids, is summarized briefly in Fig. 3.5. Here it will be seen that many of the common amino acids are in equilibrium with specific compounds in the Krebs cycle (pyruvate, α-ketoglutarate, or oxalacetate) and extensive research has shown that for the most part the specific amino acid dehydrogenases, as well as the transaminases necessary to produce these key intermediates, are located in the mitochondria.

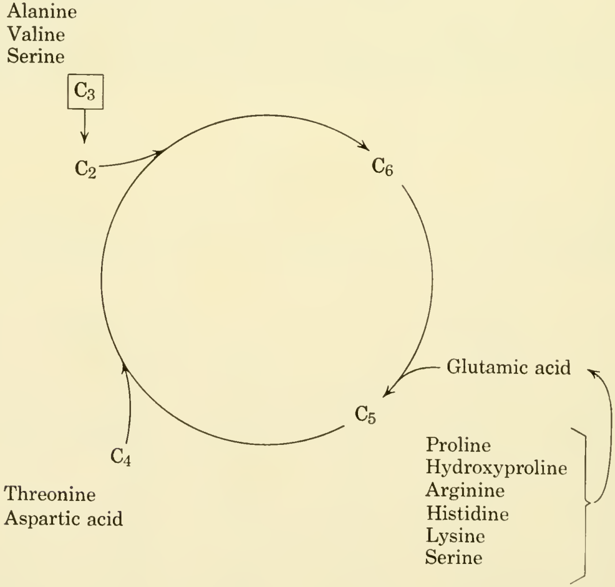

Fig. 3.5. Protein degradation and the TCA cycle.

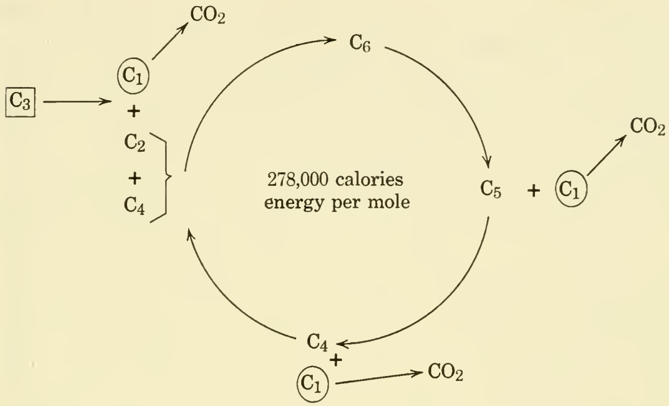

Fig. 3.6 The Krebs cycle—an energy-producing pathway.

These experiments demonstrate what was said before about the value of a concept: the idea gradually developed that the mitochondria, *and the mitochondria alone,* could furnish the crucial enzymes necessary for the oxidative breakdown of the major foodstuffs. The scheme in Fig. 3.6 formalizes this; the Krebs cycle may also be considered as an energy cycle, whose enzymes reside in the mitochondria —as Claude has said, these are the power plants of the cell. We will also see how concepts may become top-heavy; i.e., as students of comparative biochemistry added their findings during the 1940's to the pool of knowledge, namely that virtually *every* organism examined possessed an active, functioning Krebs cycle, the notion began to prevail that this pathway was the "prairie fire" of *all* terminal oxidation.

That the importance of the Krebs cycle may have been oversold to biochemists (or oversubscribed by them) is revealed in their reaction to the discovery of the Zwischenferment enzyme, or the "direct" oxidation of glucose. This pathway was discovered independently by Warburg, Lipmann, and by Dickens; it referred to the conversion of glucose-6-PO_4 to pentose phosphate, using (usually) TPN as coenzyme. This work was done in 1935–1936 (10–12); yet some fifteen years passed before most of the workers in the field appreciated its significance, although Engelhardt and Barkhash (13), as well as Dickens (14), felt that the new oxidation of glucose might be charted by quite a different pathway from the glycolysis–Krebs cycle sequence. In retrospect, it seems that the finding of a mechanism that accounted for the formation of pentose satisfied the workers of the day; certainly few people appreciated the possibility that the "direct" oxidation of glucose might take on major importance, since the Krebs cycle seemed adequately to account for the terminal oxidation of sugars.

The findings of Claude's successors merely solidified this view: the discovery that pentose phosphate could give rise to sedoheptulose phosphate and tetrose phosphate was brushed aside, and instead the new findings were emphasized for their simultaneous formation of triose phosphate. The latter compound seemed important because it gave rise easily to pyruvate, which in turn semed to provide a ready entree to the Krebs cycle; it appeared, in short, as if the excess pentose were simply being shunted into the Krebs cycle via triose phosphate, and the name "hexose monophosphate shunt" became for a time a popular term to alternately describe the "direct" oxidation of glucose.

The work of Horecker and Racker and their respective colleagues (15–17) changed this concept, for they discovered that the enzymes involved were not of the nature of a shunt mechanism at all, but instead constituted an intact system that might be considered completely separately. Although triose phosphate is formed in the transketolase reaction, the consumption of this metabolite is assured if transaldolase is also functional. Although very recent years have seen the determination that triose phosphate may feed into either the Krebs cycle or the pentose cycle or both (Fig. 2.10), it is clear from Figs. 2.2 and 2.3 that these cycles need not be interdependent; operation of the pentose pathway can under certain circumstances lead to quantitative production of CO_2, as discussed in Chapter 2.

In attempting to assess the over-all importance of the pentose cycle, a logical first question might be raised regarding the ubiquity of this complex of enzymes in living tissue. Several laboratories, including our own, have sought to demonstrate its activity in a variety of organisms, with generally complete success. In addition to the isotopic evidence cited in the previous chapter, enzymes of the pentose cycle have been found in higher animals (18–32), insects (33–37), marine organisms (38, 39), worms (40, 41), higher plants (42–48), yeast and other fungi (49–58), as well as bacteria (59–63). The enzymes of this complex seem, in short, to be at least as widespread as those of the Krebs cycle.

The demonstration of a metabolic pathway in an organism is fairly complicated. To carry out a complete search for the Krebs cycle in a new organism, for example, would probably involve at least five sets of operations: (*a*) attempts to obtain complete oxidation of each intermediate; (*b*) search for the individual enzymes, with (*c*) enough purification of each so that the suspected intermediates may accumulate and their formation be proved; (*d*) use of certain inhibitors, e.g., malonate, to permit the recognition of intermediates; and (*e*) the use of one or more isotopically labeled substrates to determine whether the intramolecular distribution of products is in agreement with expectations. Likewise, a search for the pentose cycle would follow this general outline, except for the use of specific inhibitors; there are no good ones that have been discovered that have an exclusive effect on the pentose cycle.

We have carried out this detailed type of investigation in a few situations; but I think you will agree that if one wishes to survey a large number of new organisms, progress by this means will be painfully slow. As opposed to this rigorous method of investigation, we usually use a more "vigorous" one, in which two general operations are employed: (*a*) demonstration of the presence of the dehydrogenases for G-6-P and 6-PGA; and (*b*) measurement of the *non-oxidative* disappearance of ribose, followed by a search for the transient formation of sedoheptulose and triose phosphates, and the somewhat delayed accumulation of hexose phosphate. This type of behavior is noted in Fig. 3.7, which is

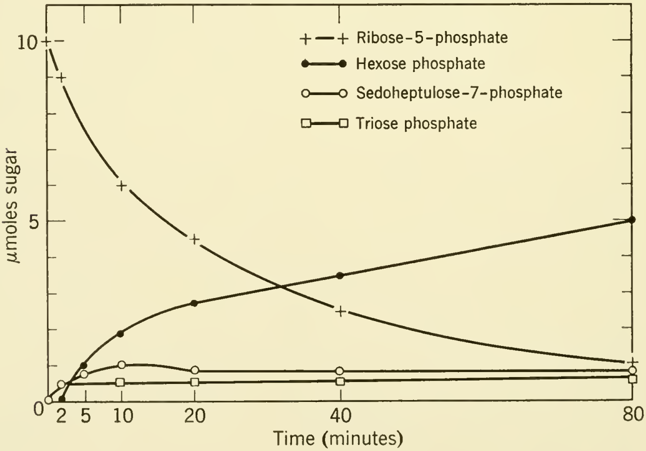

Fig. 3.7. Non-oxidative disappearance of added pentose from extracts of pea aphid. Evidence for the pentose cycle in soluble extracts of the pea aphid.

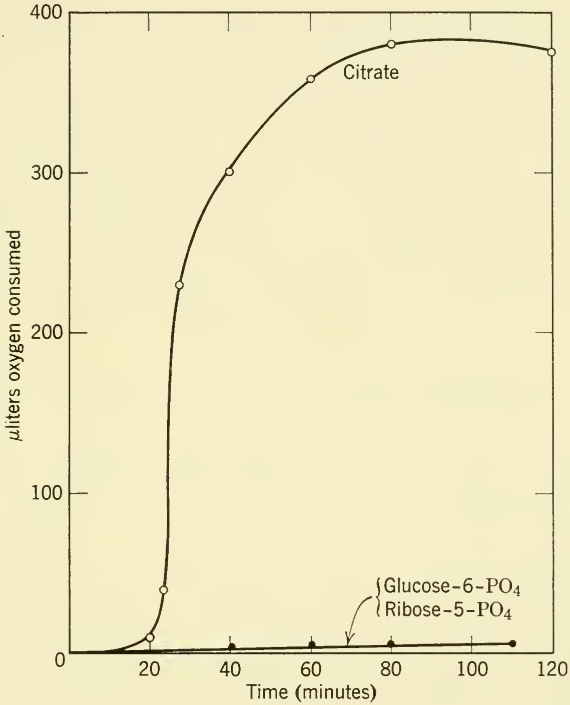

Fig. 3.8. Oxidations in rabbit liver mitochondria.

a diagram of these transformations in the pea aphid. The accumulation of sugars proceeds as outlined in accordance with expectations based on operation of the cycle.

In this connection, it seems appropriate to point out that merely to find the individual enzymes of a pathway in question is not enough to establish the operation of the pathway. The enzymes formerly regarded as "belonging" to glycolysis, for example, are, with the exception of phosphofructokinase, all shared by either the pentose cycle or the Entner-Doudoroff pathway.

A second question to which we have sought an answer is that of intracellular location of the respective enzymes. Whereas we have seen how the Krebs cycle complex is located in the mitochondria, Fig. 3.8 shows that this is obviously not true of the enzymes dissimilating G-6-P or R-5-P. Citrate added to this preparation is oxidized over 70% of theory, thus assuring the integrity of the preparation. In Fig. 3.9, the disappearance of pentose and accumu-

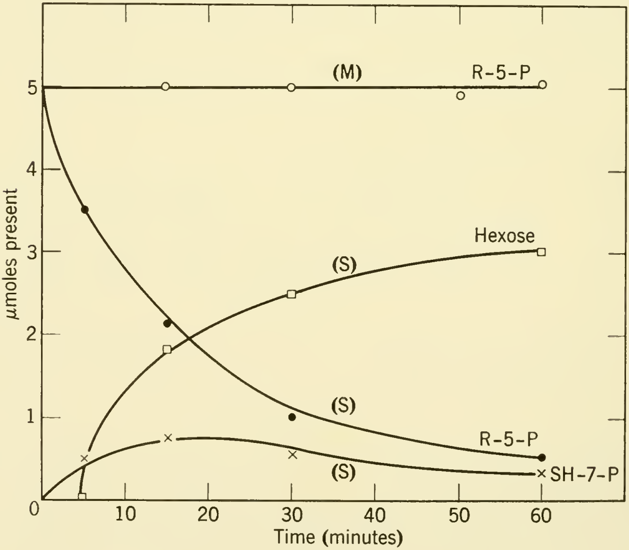

Fig. 3.9. Pentose cycle reactions in rabbit kidney fractions (nonoxidative).

M = mitochondria (residue, washed three times with isotonic KCl)
S = kidney fraction, soluble at 105,000g

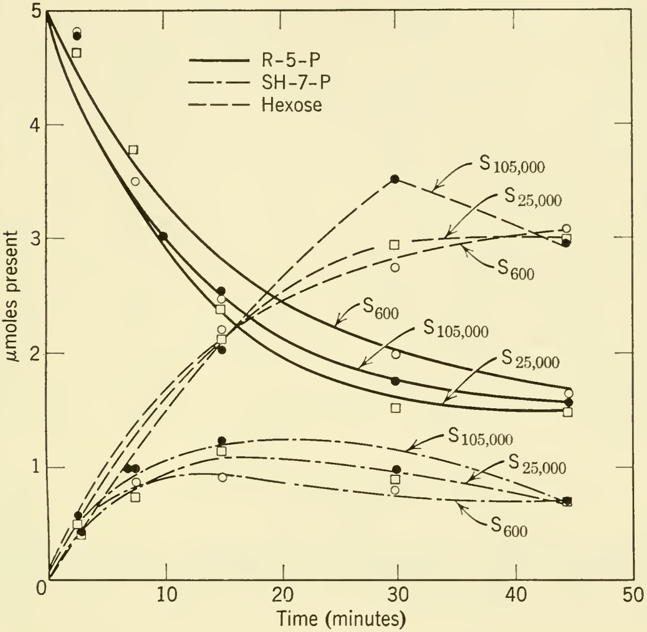

Fig. 3.10. Disappearance of added pentose in rabbit kidney (non-oxidative).

$S_{105,000}$ = soluble after centrifuging 1 hour at 105,000g
$S_{25,000}$ = soluble after centrifuging 30 minutes at 25,000g
S_{600} = soluble after centrifuging 20 minutes at 600g

lation non-oxidatively of SH-7-P and hexoses are seen to be associated with the fraction soluble (in a centrifugal field of 105,000*g*); in other words, the fraction of the cytoplasm from which both the mitochondria and microsomes have been removed. The $S_{105,000}$ fraction appears to be as active as the S_{600} or $S_{25,000}$ fractions, which contain either microsomes ($S_{25,000}$) or also the mitochondria (S_{600}) (Fig. 3.10).

This has been verified quantitatively (24). When the gravitational field is increased to 144,000*g*, all the pentose cycle enzymes are precipitated together (not shown); for this reason we are often tempted to refer to the pentose cycle as a complex; and although it may not be safe to draw firm conclusions on such indirect evidence, nonetheless we are taking as a working hypothesis the view that the entire complex may be an intact submicroscopic unit within the cell.

These two major dissimilation pathways thus appear to exist side by side in different parts of the cell. An interesting point would be the determination of the control agents that may be operating *in vivo* to direct the substrate into one or the other pathway. This is as yet un-

TABLE 3.4

Effect of Pyridine Nucleotides on Specific Activity of $C^{14}O_2$ Arising from Labeled Glucose in Pig Heart Homogenates

	C_1'/C_6' *		
Size	No Pyridine Nucleotides	DPN	TPN
Adult	1.00	1.02	2.29
Fetal (5.5 in.)	1.09	1.47	14.24

Flask components: 460 μmoles of KCl; 20 μmoles of phosphate buffer, pH 7.4; 200 μmoles of nicotinamide; 10 μmoles of $MgCl_2$; 0.14 μmole of cytochrome c; 10 μmoles of ATP (K salt); 10 μmoles of glucose, 3 μmoles of pyridine nucleotide; 2 ml. of homogenate in tris buffer; 0.3 μc. of labeled glucose. The volume was 4.15 ml.; the temperature, 37°; and the time, 5 hours.

$$^{*}\ \frac{C_1'}{C_6'} = \frac{\text{specific activity of } C^{14}O_2 \text{ from glucose-1-}C^{14}}{\text{specific activity of } C^{14}O_2 \text{ from glucose-6-}C^{14}}$$

After Jolley, Cheldelin, and Newburgh (29).

certain, but *in vitro* experiments have pointed to the influence of cofactors such as Mg^{2+}, DPT, and pyridine nucleotides. We have already discussed some of these in the first lecture, where in *A. suboxydans* normally all the carbohydrate that is broken down to CO_2 and H_2O traverses the pentose cycle; but if Dowex adsorption is used to remove Mg, DPT, and the pyridine nucleotides, then re-addition of DPN allows some glycolytic reactions to occur. In the developing foetus, Jolley et al. (29) have found that increasing the concentration of TPN can greatly increase the ratio of pentose cycle "traffic" to the glycolysis–Krebs cycle route. Table 3.4 shows that the oxidation of glucose carbon 1 compared to C-6 in pig foetus hearts is increased more than ten-fold by adding extra TPN to the medium. Although this effect is greatest with homogenates, where the influence of TPN might be expected to be felt to a relatively larger extent, the effect has also been demonstrated in whole perfused hearts; Table 3.5 describes this, where added TPN directs a doubling of the normal ratio of oxidation of C-1 compared to C-6. The effect is specific for TPN, and for the oxidation on the first carbon, as may be seen from the table in experiments with DPN. This effect of TPN on oxidation routes has also been noted by Wenner and Weinhouse (64), working with rat liver systems.

This last point gives rise to a third question: that of the share of total cellular oxidations that may normally be carried by the pentose cycle. This was discussed in Chapter 2; it is apparently not a large figure in most organisms, yet it is a significant amount. However, the *reductive* pentose cycle may also be important, as is suggested by at least three different experiments that are recorded in the literature.

TABLE 3.5

Effect of Pyridine Nucleotide on $C^{14}O_2$ Production from Labeled Glucose by Perfused Adult Rat Hearts

Substrate	Pyridine Nucleotide	Ratio *
Glucose-1-C^{14}	TPN	2.29
Glucose-1-C^{14}	TPN	2.54
Glucose-6-C^{14}	TPN	1.39
Glucose-6-C^{14}	TPN	1.00
Glucose-1-C^{14}	DPN	1.32
Glucose-6-C^{14}	DPN	1.39

Components per liter of medium: 140 mmoles of NaCl; 5.4 mmoles of KCl, 3 mmoles of $CaCl_2$; 80 μmoles of nicotinamide; 900 μmoles of phosphate buffer, pH 7.2; 1.15 mmoles of barbital buffer, pH 7.2. The temperature was 37°. CO_2 samples were withdrawn at 30-minute intervals over a 5-hour period. The pyridine nucleotide concentration was 174 μmoles per liter.

$$* \text{ Ratio} = \frac{\left\{\begin{array}{c}\text{specific activity of } C^{14}O_2 \text{ from heart}\\ \text{with pyridine nucleotide}\end{array}\right\}}{\left\{\begin{array}{c}\text{specific activity of } C^{14}O_2 \text{ from heart}\\ \text{without pyridine nucleotide}\end{array}\right\}}$$

After Jolley, Cheldelin, and Newburgh (29).

1. The first observations pointing to a synthetic role for the pentose cycle relate to the obvious need for ribose in nucleotide synthesis, and for shikimic acid. Though the biosynthesis of ribose would appear to be most simply effected by the action of G-6-P and 6-PGA dehydrogenases, indications are that this may not be the normal route. Bernstein (65), in a study of the intramolecular distribution of C^{14} in nucleotides of chicks fed labeled acetate, glycine, and formate, found it impossible to relate his findings to any oxidative scheme then known, but later

Horecker and Mehler (66) showed that Bernstein's results were in agreement with expectations from the transaldolase-transketolase sequence operating *in reverse.*

2. In higher plants, a close relative of the cycle, ribulose 1,5-diphosphate, acts as a primary acceptor of CO_2 (67, 68); the subsequent reactions leading to polysaccharide synthesis appear to employ the reverse, or reductive pentose cycle (69):

$$\begin{aligned}
&3\ \text{Pentose-P} + 3\ \text{ATP} \rightarrow 3\ \text{Ru-di-P} + 3\ \text{ADP}\\
&3\ \text{Ru-di-P} + 3CO_2 + 3H_2O \rightarrow 6\ \text{P-glycerate}\\
&6\ \text{P-glycerate} + 6\ \text{ATP} \rightarrow 6\ \text{di-P glycerate} + 6\ \text{ADP}\\
&6\ \text{di-P glycerate} + 6\ \text{TPNH} + 6\ H^+\\
&\qquad\qquad \rightleftharpoons 6\ \text{GLA-P} + 6\ \text{TPN}^+ + 6\ PO_4^{3-}\\
&2\ \text{GLA-P} \rightleftharpoons 2\ \text{DHA-P}\\
&2\ \text{GLA-P} + 2\ \text{DHA-P} \rightleftharpoons 2\ \text{F-1, 6-di P}\\
&2\ \text{F1,6-di P} + 2H_2O \rightarrow 2\ \text{F-6-P} + 2\ \text{Pi}\\
&\ \text{F-6-P} + \text{GLA-P} \rightleftharpoons \text{Xu-5-P} + \text{E-4-P}\\
&\ \text{F-6-P} + \text{E-4-P} \rightleftharpoons \text{SH-7-P} + \text{GLA-P}\\
&\ \text{SH-7-P} + \text{GLA-P} \rightleftharpoons 2\ \text{pentose-P}
\end{aligned}$$

$$\begin{aligned}
\text{Sum: } &3\ CO_2 + 9\ \text{ATP} + 5\ H_2O + 6\ \text{TPNH} + 6\ H^+\\
&\qquad \rightarrow \text{GLA-P} + 9\ \text{ADP} + 6\ \text{TPN}^+ + 8\ PO_4^{3-}
\end{aligned}$$

3. A third experiment which points to a synthetic role for the pentose cycle is the finding by Jolley et al. (29) that this complex of enzymes is much more active in the mammalian foetus than in the adult, and is most active in the very young embryo (about one-fourth of the total glucose oxidation appears to proceed by phosphogluconate cleavage). There is a gradation toward adult characteristics which is reached about three-fourths of the way through the gestation period. From this point onward the metabolic "traffic" in the heart of the foetus becomes vir-

tually exclusively one following glycolysis and the Krebs cycle, which is maintained throughout post-partum life. It is not understood why phosphogluconate cleavage disappears altogether in the late foetal stages; the need for rapid synthesis is much reduced at this point as compared to the situation in the earlier foetus, and it seems even possible that the synthesis observed in a young animal is so small (relative to oxidation) as to escape measurement by the methods used.

Finally, as suggested in the review by Horecker and Hiatt (70) a possible role of the pentose cycle (here operating clockwise, or in the forward direction) may be to supply TPNH to the system. In many species, the pentose cycle is TPN-specific; glycolysis and Krebs cycle oxidations are, on the other hand, largely DPN-requiring (a possible exception in some systems is isocitric dehydrogenase) so that processes such as fatty acid synthesis may derive significant amounts of needed TPNH from phosphogluconate cleavage.

CONCLUSION

Our ideas about carbohydrate metabolism have undergone considerable shifting during the past quarter century, as glycolysis, the Krebs cycle, and the pentose cycle have successively emerged as major pathways. Each of these appears to contribute to the breakdown of carbohydrates; in selected organisms, major use appears to be made of one or another of these pathways. In many organisms, however, a strong case appears to be developing for the employment of the pentose cycle largely as a synthetic apparatus. Time and the diligence of researchers in this area will determine whether overlapping may occur with other pathways yet undiscovered. Meanwhile, the three major routes presently recognized seem to fill the conceptual roles required for oxidation or synthesis, as well as the interchange of metabolites that is convenient to the over-all economy of the cell.

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INDEX

Italic page numbers indicate tables and figures.